AICPE
Associazione Italiana Chirurgia Plastica Estetica

Research

Autologous Cryopreserved Adipose Tissue Using an Innovative Technique: An In Vitro Biological Characterization

Carlo Ventura, MD, PhD; Elena Bondioli, BD; Roy de Vita, MD; Gino Rigotti, MD; Federico Morigi, PhD; Francesca Scarpellini, MD; Francesca Di Fede; Alessandro Nanni-Costa, MD; and Davide Melandri, MD

Aesthetic Surgery Journal
2025, Vol 45(1) NP16–NP24
Editorial Decision date: August 29, 2024; online publish-ahead-of-print September 20, 2024.

https://doi.org/10.1093/asj/sjae192
www.aestheticsurgeryjournal.com

OXFORD
UNIVERSITY PRESS

Abstract

Background: Utilization of autologous adipose tissue transplantation in plastic and orthopedic surgery such as breast reconstruction and intra-articular injection has become an attractive surgical treatment with satisfactory clinical outcomes. Nevertheless, repeated liposuctions necessary to harvest fatty tissue, normally performed with sedation or general anesthesia, may represent a noteworthy concern.

Objectives: The aim of this study was to demonstrate through an in vitro characterization the validity of the surgical option of cryopreserved autologous adipose tissue harvested in a single shot for repeated graft transfer in breast reconstruction without impairment of cell viability and sterility.

Methods: Adipose tissue was collected by standard liposuction from patients who needed numerous fat grafting procedures for breast reconstruction. According to an innovative and patented cryopreservation method, autologous adipose tissue was subsequently fractioned in a sterile bag system and frozen at the RER Tissue Bank of the Emilia Romagna Region. Each graft was evaluated for sterility and cell viability immediately after harvesting, and 1, 3, 6, 12, and preliminarily 18 months after cryopreservation and thawing.

Results: In vitro results showed that after processing, middle-term and long-term cryopreservation, and subsequent thawing, autologous cryopreserved adipose tissue retained absence of bacterial contamination, high cellular viability, and unmodified histomorphological properties, thereby ensuring maintenance of the stromal vascular niche and the filling properties in different multistep surgical procedures.

Conclusions: In vitro study and sterility assessment showed that autologous cryopreserved adipose tissue grafting is a safe procedure, making it possible to avoid multiple liposuction surgery. No impairment of sterility, cell viability, or morphology was observed over time.

Over the past decade, use of autologous adipose tissue as a tissue graft has spread rapidly in different surgical protocols for treatment of several degenerative and traumatic human tissue diseases. The reason why autologous fat in reconstructive surgery is widespread lies in its capacity to maintain different cell lineages, such as adipocytes, preadipocytes, and their precursors of mesodermal origin, as it has been demonstrated since the 2000s.[1-4] In addition to the above-mentioned mesenchymal content, it has been demonstrated that adipose tissue includes endothelial precursor cells that may exhibit hematopoietic activity.[5]

Human autologous fat grafting (HAFG) is in general employed for the treatment of multifaceted fields that need tissue augmentation, from breast reconstructive surgery to aesthetic surgery, and also is becoming popular and common in orthopedics and sport medicine.[6-11]

HAFG is considered an emerging therapeutic option for challenging wound treatment as a consequence of peripheral vasculopathy that leads to chronic wound onset, for therapeutic angiogenesis in patients with critical limb ischemia or diabetic foot, and in acute or chronic wound treatment.[12-17] More recently, HAFG has been investigated for treatment of complications related to Crohn's disease, showing good clinical outcomes in curing anoperineal fistulas, with a meaningfully clinical improvement after in situ injection of autologous HAFG or allogeneic adipose tissue–derived mesenchymal stem cells (ADSCs).[18-20]

Most importantly, to preserve the bioactivity of HAFG and its components, the stromal vascular fraction (SVF), encompassing

Dr Ventura is full professor of molecular biology, Istituto Nazionale Biostrutture e Biosistemi (INBB), School of Medicine of the University of Bologna, Bologna, Italy. Dr Bondioli is technical director, Cell Factory of Skin Tissue Bank of Emilia Romagna Region, Cesena, Italy. Dr de Vita is chief, Department of Plastic and Reconstructive Surgery, Regina Elena National Cancer Institute, Rome, Italy. Dr Rigotti is head of the Lipotech Department, San Francesco Hospital, Verona, Italy. Dr Morigi is a biologist and Dr Scarpellini is a pathologist, Department of Clinical Pathology, O.U. Pathological Anatomy, Bufalini Hospital, Cesena, Italy. Ms Di Fede is a clinical data manager consultant in private practice, Milano, Italy. Dr Nanni-Costa is a scientific consultant in private practice, Milano, Italy. Dr Melandri is a dermatologist, Department of Surgery and Trauma Center, O.U. Burn Center and Dermatology, Bufalini Hospital, Cesena, Italy.

Corresponding Author:
Dr Carlo Ventura, Via Irnerio, 49, Bologna, BO 40126, Italy.
E-mail: carlo.ventura@unibo.it

adipocytes, preadipocytes, pericytes, and ADSCs, an increasing number of systems have been developed to process or isolate autologous adipose tissue elements, overcoming donor variations, risk for infections, and unpredictability of the final product. These procedures, carried out with specific processing devices, follow defined requirements when processing the harvested fat, particularly concerning minimal tissue manipulation. To date, many nonenzymatic isolation methods and devices (the enzymatic isolation falls within the high manipulation process, with regulatory implications) have been developed to process human adipose tissue, maintaining the integrity of both the SVF and the tissue microenvironment (the stromal vascular niche), ensuring full compliance with the minimal manipulation regulatory requirements of human cells, tissues, and cellular/tissue-based transplantation. In Italy, the clinical use of adipose tissue is regulated by the Legislative Decree (DLGS) 191/2007, which has been adopted pursuant to European Directive 23/2004 EC on standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells. Moreover, the subsequent Legislative Decree, DLGS 16/2010, implements the technical requirements in compliance with European Directives 2006/17 EC and 2006/86 EC on traceability and notification of serious adverse events (SAE), including specific technical requirements according to Directive 23/2004 EC.

The above-mentioned nonenzymatic processing systems apply mechanical forces or filtering steps by means of a semipermeable membrane to separate the cells or fat nanoclusters from oily residues and hematic components, minimizing cellular stress and preserving the integrity of the extracellular matrix (ECM) with a sound efficacy, even with a lower cell yield than enzymatic-dependent techniques.[21,22]

In recent years, through a multidisciplinary approach, breast reconstructive surgery has become a full-fledged part of breast cancer treatment and has greatly contributed to the improvement of the patient's quality of life. It is therefore considered an integral part of the diagnostic and therapeutic pathway for the treatment of breast cancer. In the mid-1990s, the introduction and use of HAFG in breast reconstruction was considered an elective treatment at a single surgical time, thanks to the supply of "new tissue," limiting the need for prostheses.[23] In the 2000s, HAFG as lipofilling in reconstructive mastoplasty procedures became increasingly common, and the clinical scientific literature consistently reported satisfactory outcomes with HAGF, up to 7 years of follow-up.[24-27] There was also success with grafting megavolumes of fat, and even adopting hybrid surgical approaches involving the implantation of a prosthesis associated with an autograft of adipose tissue.[28,29] Moreover, some randomized clinical trials reported that autologous fat transfer (AFT) was estimated to be more cost effective in the long-term follow-up period because no additional surgeries were necessary for this group, in comparison to the control group that received implants.[30] The procedure was safe, with no or marginal effects on the probability of postmastectomy locoregional recurrence of breast cancer.[31] Nevertheless, the standard surgical protocol of reconstructive mastoplasty with AFT usually required multiple treatments, and the patient underwent repeated surgical sessions to achieve satisfactory clinical outcomes. Furthermore, the procedure had to be achieved quickly enough to preserve sterility and avoid necrosis, considering its poor vascularization and also absorption of the tissue, which could adversely affect the final desired clinical outcome.[32-34] The patients who had undergone fat grafting with necessary simultaneous repeated liposuction perceived discomfort, because this procedure was the most invasive and painful phase of the surgical protocol and required at least major sedation with the presence of an anesthesiologist, and finally requiring medical compression garments for 3 weeks to avoid morbidity at the donor site (ie, swelling, pain, or bruising). Other limitations and discomfort of that procedure included increased time of surgery, home medical care service, higher medical costs, greater loss of working days, and, finally, the precious fat derivative after the initial surgery was discharged.[35,36] To overcome the limitations and relative therapeutic disadvantages of that procedure, several articles have recently been published on cryopreserving adipose tissue under certain conditions and appropriate treatments, such as with cryoprotectant agents (CPAs) and adjustments of cooling rate and thawing time, to protect the tissue. In general, these storage procedures have been shown not to meaningfully modify the ADSCs and associated molecular marker components biologically, keeping the differentiative and regenerative power intact and allowing fractionation of the volume of the harvested fat, making it available for subsequent—even after an extended period—multiple grafts to complete the surgical protocol.[37] The scientific rationale of this approach has been experimentally demonstrated by the isolation and preservation of ADSCs in unmanipulated and long-term cryopreserved human adipose tissue without additional collagenase isolation. The investigators demonstrated that a longer cryopreservation of adipose tissue may impact negatively on cell viability in comparison to short-term cryostorage. However, they demonstrated that isolated ADSCs derived from whole cryopreserved fat after a long time had a higher proliferative capacity, also maintaining their multipotency in the elderly population, allowing serial fat grafting. In fact, the ADSC phenotypes were confirmed by fluorescence-activated cell sorting (FACS) analysis of the specific markers of human mesenchymal stem cells. The results showed that the great majority of adipose stromal cells (ASCs) were CD105+ and CD45−. Furthermore, it was observed that ASCs isolated at all cryopreservation times were capable of differentiating into both osteogenic and adipogenic phenotypes, as demonstrated by positive staining with alkaline phosphatase and Oil Red O, respectively.[38] Nevertheless, a standard cryopreservation procedure to guarantee a high level of viability of adipocytes and other cellular elements in the SVF is still currently debated, particularly concerning the cryoprotective agent variables. Therefore, cryopreservation and cryobiology remain topics more relevant than ever, and represent a key requirement for a clinically successful procedure.[36,39]

Autologous adipose tissue processing and cryopreservation according to a standardized, innovative, and patented methodology make it possible to obtain a product in which the overall tissue matrix, the microenvironmental structure, and the resident cells residing within remain unaltered, without damage, preserving their original physiological, morphological, and viability characteristics.[35,40] With this method, the resulting final product is minimally manipulated cryopreserved tissue specifically for autograft use. Furthermore, most importantly, fat grafting without simultaneous liposuction can be carried out under local anesthesia or a nerve block (it is feasible through light sedation), and the treatment can be performed in outpatients without hospitalization, requiring no rehabilitation. It is ultimately less expensive and more effective in addressing the current challenge of long hospital waiting lists.

Therefore, the specific aim of this in vitro investigation was to demonstrate the validity of the above-mentioned innovative method for cryopreserving and thawing autologous adipose tissue and maintaining its adipogenic properties as an effective treatment for subsequent planned breast reconstructive surgeries.

METHODS

Adipose Tissue Collection and Processing

After written informed consent was obtained, the lipoaspirate was harvested by elective liposuction from 12 consecutive patients who

underwent breast reconstruction with autologous fat grafting from July 2022 to June 2023. We conducted this investigation in compliance with the principles of the Declaration of Helsinki, and the procedure was carried out in accordance with common hospital practices at the following Italian hospitals: Department of Plastic and Reconstructive Surgery at the Regina Elena National Cancer Institute in Rome, and the San Francesco Clinic in Verona. Under local anesthesia, adipose tissue was collected in a variable volume, based on the area that needed lipofilling, from different anatomical sites (abdomen, hips, thighs, buttocks) by a plastic surgeon with a thin cannula (3-mm diameter) equipped with a blunt tip and connected to a syringe. The procedure was carried out after infiltration into the subcutaneous panniculus with Klein solution (saline solution mixed with adrenaline [final concentration of 2 μg/mL, 1:500,000] and lidocaine to a dilution of 0.02%) in a ratio of 1:1 with the volume of tissue to be collected. After 10 to 15 minutes, local anesthesia and ischemia were achieved at the donor site with limited bleeding, and the adipose tissue was harvested with the cannula connected to a syringe while negative pressure was maintained.

Suctioning was carried out at moderate speed to minimize adipocyte damage. When the syringe was filled, the cannula was removed, and another syringe was connected until the required volume of lipoaspirate was reached. Then, the lipoaspirate was left to decant in the surgical theater to reduce and eliminate any fibrous frustules present. The purified adipose tissue was then transferred into a certified ethylene vinyl acetate (EVA) bag and immediately shipped under a controlled temperature of 4°C to the RER Tissue Bank of the Emilia Romagna Region of Cesena (Italy) for cell characterization and cryopreservation.

Lipoaspirate Delivery and Cryopreservation at RER Tissue Bank

Upon delivery, an identification code containing the donor's initials was assigned to each lipoaspirate, ensuring anonymity and full traceability of the samples for this characterization. Samples were fractioned through a closed-loop system (LipoBank S.r.l., Milano, Italy) in aseptic conditions in a laminar flow cabinet (class A) in a dedicated class B good manufacturing practice (GMP)-certified room of the RER Tissue Bank.

According to the standard protocol of the RER and LipoBank cryopreservation method, the preliminary cellular characterization of fresh tissue and the cryopreservation process were completed within 48 hours of receipt of the collected lipoaspirate. Thirty minutes before freezing, a cryoprotective agent solution (0.5 M DMSO/0.2 M trehalose) was added drop by drop to a 15-mL test tube containing fresh adipose tissue at a volume ratio of 1:1 and then transferred into 0.5-mL sealed cryopreservation-specific paillettes. With a controlled blast chiller, the tubes were subjected to slow and controlled freezing, lowering the temperature by 1°C per minute to −18°C, then they were transferred into liquid nitrogen vapor at a temperature of approximately −195°C.

Sterility control and cell characterization were achieved for each fresh sample, and thawing was performed after 1, 3, 6, 12, and preliminarily 18 months of cryopreservation. Microbiological tests demonstrated maintenance of sterility of the lipoaspirate over time.

Thawing of Frozen Adipose Tissue

The thawing of autologous adipose tissue was carried out in the aseptic and GMP-certified environment of the cell factory of the RER in compliance with the current regulations of the National Transplant Center. The process was completed with the Conformite Europeenne (CE)-marked procedural kit LIPOWASH (LipoBank) specifically dedicated to the procedure of thawing and washing adipose tissue for subsequent clinical use.

The samples of adipose tissue inside the packages taken out of liquid nitrogen were thawed with a thermostatic bath (model 20-TW20-Seneco; Julabo S.r.l., Italy) at a temperature of 37°C for 1 minute under the laminar flow hood. Then they were placed into containers to maintain a temperature of 4°C. Finally, under a sterile hood, the thawing procedure was completed with an appropriate washing of the adipose graft.

Approximately 30 minutes before thawing, the following 3 washing solutions at decreasing concentrations of DMSO/saline in trehalose/saline were prepared in a volume of 6 mL each: 0.3 M of DMSO + 0.2 M of trehalose (solution 1); 0.1 M of DMSO + 0.2 M of trehalose (solution 2); and 0.2 M of trehalose (solution 3). Solutions were maintained at 4°C under sterile conditions in a clean room.

With sterile Pasteur pipettes, the adipose tissue was then transferred from the tube containing washing solution 1 into another tube containing washing solution 2, where the adipose tissue was further mixed and transferred into a third tube containing washing solution 3, where it was again gently stirred. Finally, the adipose tissue suspension from the third tube was transferred into a tube containing 6 mL of complete cell culture medium, consisting of Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific; Waltham, MA) low glucose, 1% L-glutamine, 1% penicillin-streptomycin, and 20% fetal bovine serum. After an overnight incubation at 37°C with 5% CO_2, the adipose tissue was subjected to the viability analyses, metabolic activity assessment, and tissue architecture evaluation.

Samples of fresh lipoaspirate and samples of tissue thawed after intervals of 1 month, 3 months, 6 months, and 12 months, respectively, were assessed in vitro to validate the cryopreservation process. The following tests were carried out in triplicate: sterility tests, endotoxin activity assays, cell counts, cell viability assays by Cell Proliferation Kit I (MTT) test and LIVE/DEAD test (Thermo Fisher Scientific), histology, and immunohistochemistry.

Sterility Assessment

Sterility Test With Selective Culture Medium

The sterility assay consisted of seeding in aseptic conditions the samples of fresh autologous adipose tissue, after processing in a class B GMP-certified environment, and thawed adipose tissue in plates of TSA (tryptic soy agar; Agricons Ricerche, Padova, Italy) under a class A laminar flow hood within the dedicated class B GMP-certified environment. The plates were then incubated in an incubator (Thermo Fisher Scientific) at a temperature of 30°C to 35°C for 5 days. The appearance of colonies at any point during the 5 days was scored as microbial growth present.

In the event of positive microbial assessment on a sample, after reporting the biomolecular characterization and evaluation of the microbial load to O.U. Microbiology and Virology, the RER Tissue Bank activated an "emergency procedure" defined within the standard operative procedures, which included decontamination of the adipose tissue with a washing solution containing antibiotics and antimycotics in standardized amounts for a validated contact time established as 20 minutes (expected range, minimum 15 minutes to maximum 25 minutes of contact). The decontaminating solution was added to the sample thawing procedure and therefore was totally removed from the first washing step. The BacT/ALERT (Biomerieux, Marcy l'Etoile, France) sterility test was repeated to assess the sterility of the sample, and only in the presence of a negative result,

corresponding to no pathogenic growth, was the adipose tissue considered biologically suitable for transplantation.

Sterility Test: Microbial Contamination Assay With BacT/ALERT PLUS System

With the aerobic and anaerobic BacT/ALERT iFN and iFA plus culture bottles and the BacT/ALERT Microbial Automated Detection System, additional microbial controls for bacteria, fungi, and mycobacteria were completed in duplicate, respectively, on the fresh adipose tissue after collection, adipose tissue after processing in the class B GMP-certified environment (for the purpose of cryopreservation and storage in nitrogen vapor), and adipose tissue thawed before release and clinical transplantation use, in a class B GMP-certified environment. For these assays, sample liquid was loaded into a sterile syringe with a needle and injected into the culture liquid through a rubber septum. Both the aerobic and anaerobic media bottles were kept for 7 days at 37°C. Any growth within 7 days, every 24 hours, was scored as positive, and the sterility results were finally reported in the clinical record.

Endotoxin Activity Assay: Limulus Amebocyte Lysate Test

The LAL (Limulus amebocyte lysate) test is a specific and accurate assay for the detection and quantification of gram-negative bacterial endotoxins. According to regulatory authorities, the LAL assay is a recommended test for quantifying the presence of endotoxins in drugs, devices, and tissue engineering products. The US Pharmacopoeia Bacterial Endotoxins Test (USP Bacterial Endotoxins Test) is currently the official reference test. For the present validation, it was performed to test all adipose tissue samples previously processed and cryopreserved after thawing, and on the final tissue for clinical transplant use. The LAL test was performed with the LAL kit (Pyrotell STV, Associates of Cape Cod Inc., East Falmouth, MA) according to the manufacturer's recommendations.

Cell Count, Cell Viability Assay, and Cytotoxicity

Automated Cell Count

The automated cell count was planned as a quantitative analysis of cell viability on the autologous adipose tissue previously cryopreserved, after thawing, by LipoBank method. This assay was carried out in collaboration with the Bioscience Laboratory of Emilia Romagna Institute for Cancer Care ("Dino Amadori"; IRST S.r.l., Meldola, Italy), with the TC20 Automated Cell-Counter (BIO-RAD Laboratories S.r.l., Segrate, Italy). Specifically, for the analysis of adipose tissue, the instrument was set for a cell count with a range of size 4 to 60 μm. This provided the number of total cells and the number of viable cells per mL. It also enabled a count of irregular cells because of its accuracy in declustering cells. This test was performed to assess cell viability of the thawed tissue over time.

Cell Proliferation Kit I (MTT) Test

The MTT is a standard colorimetric assay for assessing cellular metabolic activity of biological samples. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of this salt into formazan crystals by living cells, which determines mitochondrial activity. The subsequent application of substances such as glycerol, methoxyethanol, or DMSO promotes lysis of the cell membrane, resulting in precipitation of the incorporated salts, which spill into the extracellular solution with consequent pinkish-purple coloration in a very short time, with a purple color intensity proportional to the salts produced and directly related to the cellular metabolic activity of the analyzed samples. For this test, samples of patients were included and the test was performed in replicate in a multiwell plate together with negative controls. Samples of adipose tissue (1 mL) were weighed with the precision analytical balance Analytical Plus Model AP250 DE (Ohaus Corporation, NJ). Afterward, 1 mL of MTT (Sigma Aldrich, St. Louis, MO) was added to the adipose tissue, and the plate was incubated in a Hera Cell 150 incubator (Thermo Fisher Scientific, Waltham, MA) at 37°C and 5% CO_2 for 3 hours.

After this incubation period, the MTT solution was removed by aspiration and the formazan precipitates were solubilized by adding DMSO (CRYO ON AL.CHI.MI.A. S.r.l., Padova, Italy) to the tissue in a 1:1 ratio, and then the whole was transferred into cuvettes.

Twenty minutes after the addition of DMSO, a reading was taken with a UV/VIS spectrophotometer (Beckman Coulter TM DU 530 Spectrophotometer; Beckman Coulter, Brea, CA) at a wavelength of 570 nm. The viability of the adipose tissue was calculated by performing an estimate of the average percentage viability index (I.V.%) obtained from the ratio of optical density (OD) to the weight of each sample (in grams), with the formula I.V.% = OD/gram.

LIVE/DEAD Viability/Cytotoxicity

Adipose tissue samples also were analyzed for cell viability and toxicity with the Cell Viability Assay LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific) for mammalian cells. The kit provides a 2-color fluorescence cell viability assay that is based on the real-time determination of live and dead cells with 2 probes that measure well-known parameters of cell viability, intracellular esterase activity and plasma membrane integrity. The green dye highlights viable cells and the red of propidium iodide highlights suffering or dead cells.

After incubation at 37°C, the assay was conducted with a Nikon Inverted Microscope Eclipse Ti-E light microscope (Nikon Instruments, Tokyo, Japan) equipped with a DS-03 digital camera connected to a computer and operated by NIS-Elements Imaging Software, version 4.30 (Nikon Instruments). After incubation at 37°C for 30 minutes in the dark, the cells were imaged by fluorescence microscopy with an 80i Eclipse fluorescence microscope (Nikon Instruments).

Histology and Immunohistochemistry

Hematoxylin and Eosin Staining

With the collaboration of the Department of Anatomical Pathology at M. Bufalini Hospital, Cesena, Italy, histological investigation was performed on all adipose tissue specimens fresh, after processing and cryopreservation and thawing, and before clinical use to evaluate the effects of the LioBank cryopreservation technique on pattern, size, and structure—and so on adipose tissue integrity. All samples were fixed in 12% w/v formaldehyde solution with the addition of a 0.5-M phosphate buffer (Bio-optica S.p.a., Improving Pathology, Milano, Italy), then sectioned (3-μm-thick) with a microtome and stained with hematoxylin and eosin. The resulting histological slides were then analyzed, comparing the morphology of cells before cryopreservation and after thawing with a light optical microscope (Leica mod. DL-DM IL; Leica Microsystems GmbH, Wetzlar, Germany) at 10× and 40× magnification, and photographed with a computerized high-resolution digital camera (Leica DM IL LED software LAS-X, Leica Microsystems GmbH).

Immunohistochemistry

For immunohistochemical assay, sections of processed fresh and thawed adipose tissue samples were deparaffinized, rehydrated,

Table 1. Automated Cell Count

	Automated cell count	
	M	SD
Fresh ($n = 7$)	1.31×10^6	0.21×10^6
1 month ($n = 4$)	1.08×10^6	0.57×10^6
3 months ($n = 4$)	0.91×10^6	0.41×10^6
6 months ($n = 4$)	0.91×10^6	0.57×10^6
12 months ($n = 4$)	1.00×10^6	0.59×10^6

M, mean; SD, standard deviation.

Table 2. Adipocyte Viability After Short-term and Mid-term Cryopreservation and Thawing

	% Index viability	
	M	SD
Fresh ($n = 12$)	89.7	13.6
1 month ($n = 11$)	81.3	16.9
3 months ($n = 9$)	74.3	15.5
6 months ($n = 11$)	89.9	11.6
12 months ($n = 4$)	77.9	16.4

M, mean; SD, standard deviation.

fixed on slide surfaces, and incubated with primary ready-to-use mouse monoclonal antibodies (MoAb; Roche-Ventana Medical Systems, Oro Valley, AZ) for CD31+, CD34+, CD45+, and CD117+ markers. As a secondary antibody, a goat anti-mouse antibody (Roche-Ventana Medical Systems) was utilized. CD31+ was used for characterization of endothelial cells, also immunohistochemically expressed in monocytes or macrophages, megakaryocytes, and platelets and granulocytes. The presence of CD31+ was particularly of note for therapeutic usage of ADSCs, due to its modulating effects on endothelial junction activity for inflammatory or thrombotic conditions; CD34+ was remarkable for characterization of stem cells typically residing physiologically within the adipose tissue; CD45+ was amenable to identifying primarily lymphocytes and was a cytoplasmic marker; and CD117+ was suitable for characterization of hematopoietic stem cells.

Evaluation of the Stromal Vascular Niche

Fresh and thawed samples of adipose tissue were fixed and incubated with monoclonal antibodies directed to (VE)-cadherine, an extremely important adhesion molecule in maintaining the integrity and permeability characteristics of the vascular endothelium, and to CD31 (PECAM-1), an essential marker of endothelial cells that identifies these cells and tracks their distribution. These markers, highlighted by a red and a green color, respectively, make it possible to appreciate the structure of the so-called stromal vascular niche, a sort of special microenvironmental topography in which the stem cell elements and the other SVF components reside, within the context of specific vascular architectures.

Statistical Analysis

Parametric and numeric data were reported as mean ± SD. Comparisons of parametric values were performed with 1-way analysis of variance (ANOVA) and a *t* test, with statistical significance set at an α-value of $P < .05$. Statistical analysis was carried out with Statistic Kingdom Free Software (https://www.statskingdom.com).

RESULTS

Sterility Assessment

Upon receipt at the RER Tissue Bank of fresh adipose tissue samples, microbial tests for aerobic and anaerobic bacteria and fungi from culture supernatants with selective culture medium were negative. The BacT-Alert Sterility System exhibited positivity in 4 samples out of 12, however the bacteria detected were found to be normal bacterial flora of the skin. In fact, the most frequently detected pathogen was *Staphylococcus epidermidis*. The sterility control was subsequently performed for the thawed adipose tissue, before clinical use, at the following intervals: 1 month, 3 months, 6 months, and 12 months. The results of both microbiological tests (conducted simultaneously) showed no bacterial contamination at all time intervals analyzed, confirming that sterility of adipose tissue was maintained in the cryopreservation process.

Endotoxin Activity Assay: Limulus Amebocyte Lysate Test

For all samples analyzed at the same intervals as for sterility assessment, the LAL test showed the absence of endotoxin contamination (less than 10 IU/mL). Additionally, a preliminary evaluation on a limited group of samples ($n = 8$) at an 18-month interval confirmed the negative outcomes of previous time intervals.

Cell Count, Cell Viability Assay, and Cytotoxicity

Automated Cell Count

The findings of automated cell counts were available when fresh ($n = 7$), and at 1 month ($n = 4$), 3 months ($n = 4$), 6 months ($n = 4$), and 12 months ($n = 4$) after thawing, and the results showed no statistically significant differences in cell numbers/mm^3. The average numbers of viable cells were: $1.31 \pm 0.21 \times 10^6$; $1.08 \pm 0.57 \times 10^6$; $0.91 \pm 0.41 \times 10^6$; $0.91 \pm 0.57 \times 10^6$; and $1.00 \pm 0.59 \times 10^6$. No statistically significant differences were observed when comparing the groups ($P = .405$). Data are summarized in Table 1 and Figure 1.

MTT Test

Autologous adipose tissue processed and cryopreserved with this innovative technique maintained adipocyte viability after short-term and mid-term cryopreservation. The average I.V.% of viability of sample derived from fresh tissue ($n = 12$) was 89.7 ± 13.6, and at 1 month ($n = 11$), 3 months ($n = 9$), 6 months ($n = 11$), and 12 months ($n = 12$) it was 81.3 ± 16.9, 74.3 ± 15.5, 89.9 ± 11.6, and 77.9 ± 16.4, respectively. None of the comparisons between groups showed statistically significant differences ($P = .847$). Data are summarized in Table 2 and Figure 2.

LIVE/DEAD Viability/Cytotoxicity

The results obtained with fluorescence (qualitative) analysis were from a total of 4 fresh adipose tissue samples randomly selected

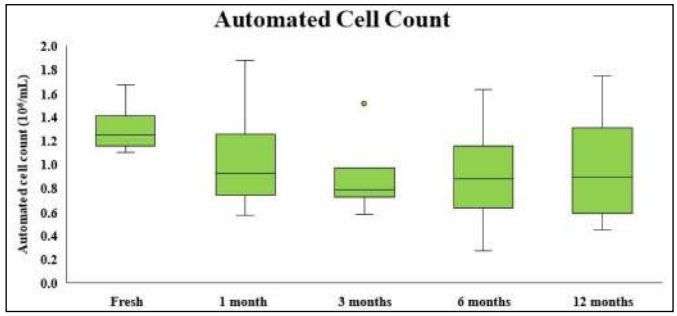

Figure 1. Automated live cell count. No statistically significant differences were observed.

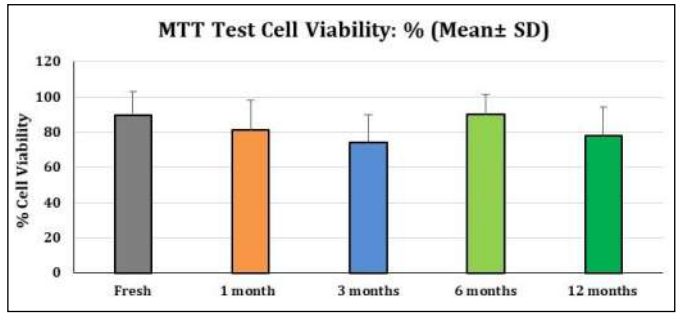

Figure 2. Mean percentage ± standard deviation of cell viability on MTT test. No statistically significant differences were observed.

and the corresponding samples thawed after cryofreezing at 1, 3, 6, 12, and 18 months respectively. This assay allowed us to highlight and distinguish viable cells (green staining) from suffering cells (red staining). The results unveiled that, although there was an intrinsic variability related to the individual patient, the profile of cell viability in the thawed tissues after cryopreservation was entirely comparable to that observed in the same fresh samples. Nevertheless, an increasing number of suffering cells were observed when tissue was thawed at 18 months (red staining). Representative images of analysis are displayed in Figure 3.

Histology and Immunohistochemistry

Hematoxylin and Eosin

On microscopic examination with hematoxylin and eosin staining, fresh samples exhibited an overall normal architectural structure, represented by rich adipocytic cellular components with normal morphology of the nuclei and the presence of some capillary vessels observed (Figure 4). The same autologous adipose tissue samples thawed after 1, 3, 6, 12, and 18 months showed an overall preserved structure characterized by the presence of numerous mature adipocytes and a few preserved capillary vessels.

Immunohistochemistry

At immunohistochemistry qualitative assessment, all tissue observed showed focal presence of well-preserved and narrowed mature endothelial cells within the vessel network (CD31+). Furthermore, CD34+ and CD45− confirmed the widespread presence of precursor endothelial cells and mesenchymal components.

All fresh and thawed adipose tissues were characterized by a rare presence of lymphocytes, as evidenced by positivity of the cytoplasmic lymphocyte marker CD45+. A very low expression of CD117+ as a marker for hematopoietic stem cells was observed. As shown in Figures 5-8, and Supplemental Figure 1 located online at https://doi.org/10.1093/asj/sjae192, cells derived from both fresh and cryopreserved tissues showed a similar distribution for all markers. In conclusion, both methods (hematoxylin and eosin histology and immunohistochemical typing) demonstrated that the procedures of harvesting, freezing, banking, and thawing did not structurally damage the adipose tissue and the viability of its cellular components.

Evaluation of the Stromal Vascular Niche

The fluorescence qualitative assessment performed with monoclonal antibodies for (VE)-cadherin and CD31 (PECAM-1) was on fresh fat graft and tissue thawed after 1 month only. The results showed that the microfragmented adipose tissue preserved the architecture of the stromal vascular niche and its components, the pericytes, endothelial cells, and mesenchymal stem cells (green staining), maintaining the integrity and permeability of the vascular endothelium (red staining) after thawing, with no detectable differences when compared with the fresh samples (Supplemental Figure 2, located online at https://doi.org/10.1093/asj/sjae192).

DISCUSSION

The present experimental in vitro characterization studies were set up to assess whether an innovative process of fractionation, cryopreservation at −196°C, and thawing of unmanipulated autologous adipose tissue could retain the biological characteristics of the fresh tissue of origin, thereby providing a worthwhile therapeutic alternative to the current procedure of breast reconstruction after mastectomy with multiple autograft lipofilling. The usual surgical procedure involves fresh fat grafting, dumping of the exceeding tissue, and a recurrent course of treatment with autologous lipoaspirate, repeating surgical sessions of liposuction, leading to a higher cost, donor site adverse events, and discomfort for the patient. Therefore, cryopreservation of the lipoaspirate derivative with subsequent thawing represents an innovative approach to autologous fat grafting in all its surgical applications—in orthopedics, cosmetics, regenerative surgery, and particularly in breast reconstructive procedures after oncologic surgery, given the peculiarity of patients who undergo invasive surgery and subsequent chemotherapy and radiation treatments.

The clinical rationale and validity of cryopreserved fat graft has been debated in the clinical and scientific community. Some authors believe that cryopreservation upholds the biological characteristics of the cryopreserved tissue when compared to the fresh tissue, such as volume and cell viability, with maintenance of cell phenotypes and the regenerative potency of the SVF where the mesenchymal cellular component nests.[40] Others believe that freezing and thawing subjects the tissue to damage, with significant reduction in cell viability, which may trigger necrosis when grafted, with loss of functional properties and regenerative potential, inducing surgeons to reflect on the therapeutic utility of freezing adipose tissue to achieve satisfactory clinical results.[41,42]

In the past, given the popularity of autologous fat grafting in cosmetic and reconstructive surgery, conventional cryopreservation techniques of lipoaspirate involved freezing the tissue at −196°C without cryoprotectant; furthermore, the protocols of thawing of the adipose tissue were animatedly discussed—for instance whether it might be more advantageous to thaw the tissue at 37°C in a bath.[43,44] More recently, several studies have described investigations of different types of cryoprotectants in an attempt to identify which one had the best reduction of toxicity in the tissue, an essential condition for maintenance of cell viability.[45] Among these studies, in a recent systematic review, even though the authors concluded that further studies to determine best standard protocols were needed, it

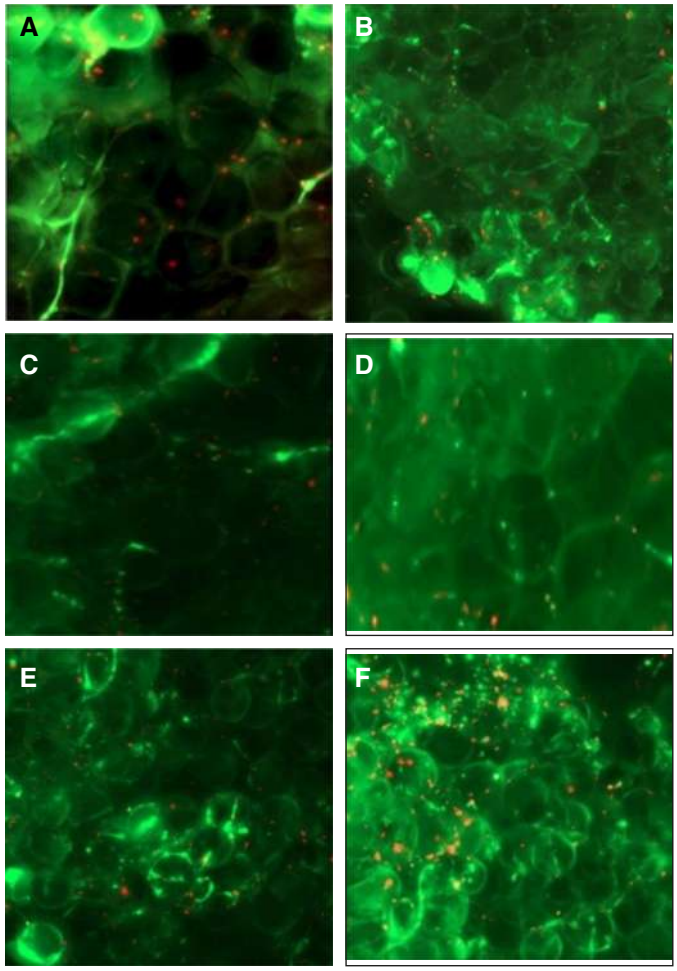

Figure 3. (A) Fresh adipose tissue. (B) Thawed after 1 month. (C) Thawed after 3 months. (D) Thawed after 6 months. (E) Thawed after 12 months. (F) Thawed after 18 months.

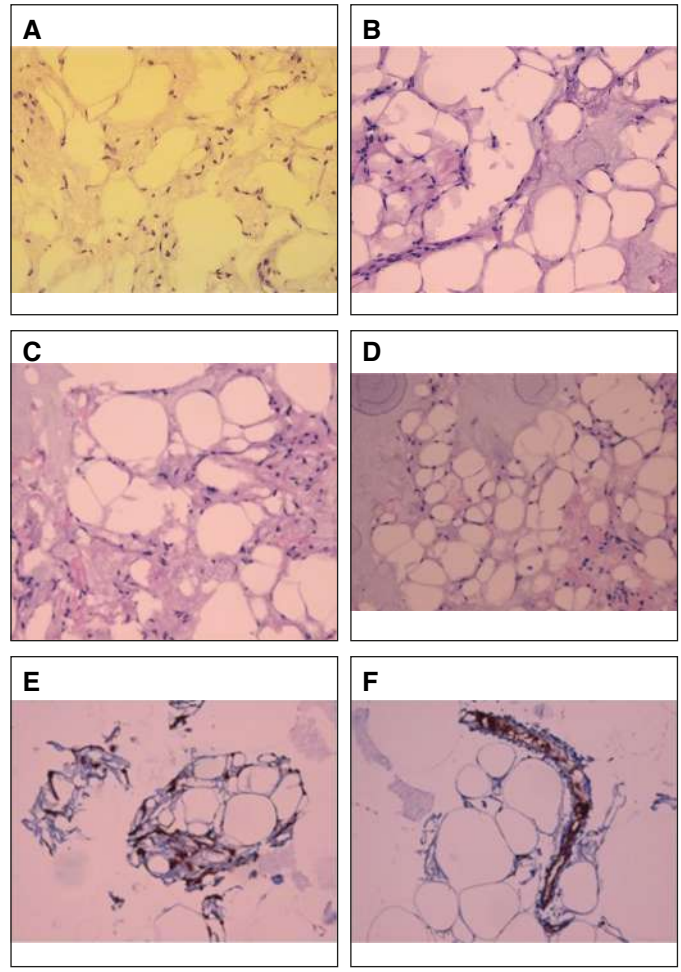

Figure 4. Histologic examination (20×) of fresh and thawed adipose tissue. (A) Fresh. (B) Thawed after 1 month. (C) Thawed after 3 months. (D) Thawed after 6 months. (E) Thawed after 12 months. (F) Thawed after 18 months.

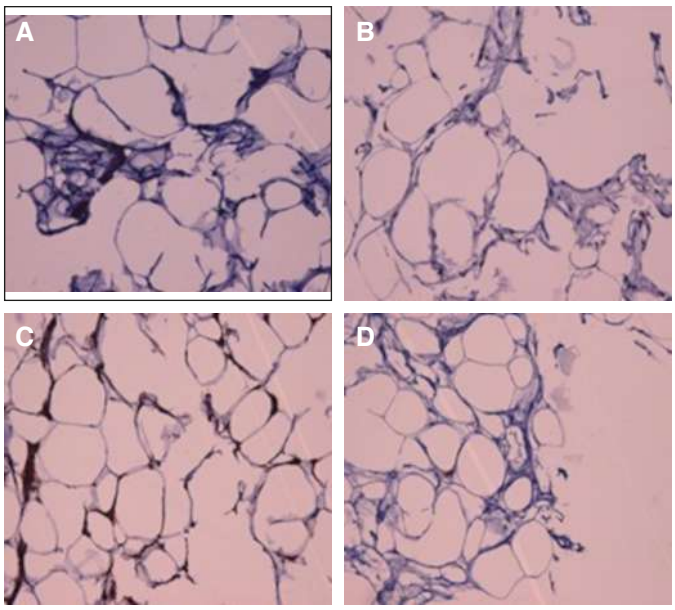

Figure 5. Immunohistochemistry images of fresh adipose tissue. (A) CD34+. (B) CD31+. (C) CD45+. (D) CD117+.

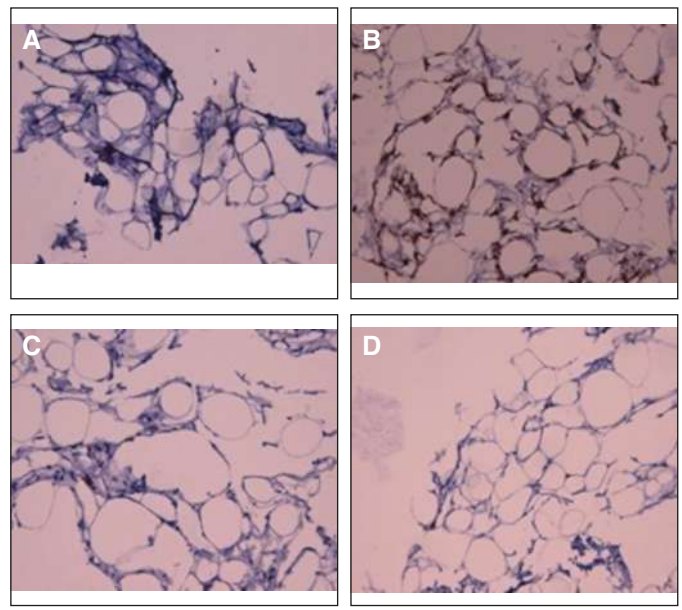

Figure 6. Immunohistochemistry images of adipose tissue thawed after 1 month. (A) CD34+. (B) CD31+. (C) CD45+. (D) CD117+.

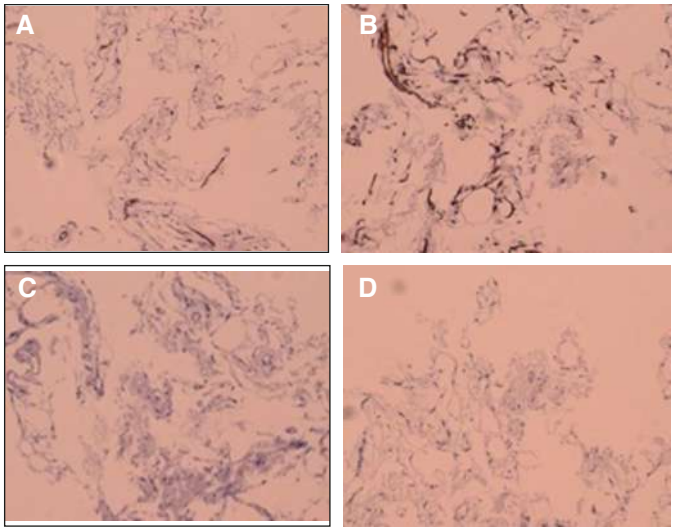

Figure 7. Immunohistochemistry images of adipose tissue thawed after 3 months. (A) CD34+. (B) CD31+. (C) CD45+. (D) CD117−.

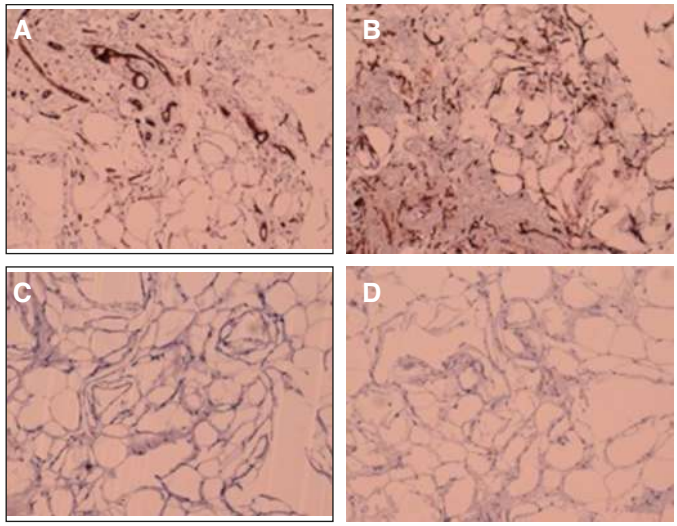

Figure 8. Immunohistochemistry images of adipose tissue thawed after 6 months. (A) CD34+. (B) CD31+. (C) CD45+. (D) CD117−.

was demonstrated that adipose tissue could be successfully cryopreserved over the long term with CPAs such as DMSO and trehalose, without compromising cell morphology and viability. These recent studies allowed improved and standardized cryopreservation protocols demonstrating that the cryopreserved adipose tissue substantially maintained the viability, cellular phenotype, and regenerative properties of the SVF over the medium- and long-term period when compared to fresh graft, ensuring safe clinical use with satisfactory clinical findings, as demonstrated in preliminary trials in humans.[46,47]

In our preliminary in vitro study, we have investigated and subsequently validated a patented method for collection and cryopreservation of autologous adipose tissue as part of a therapeutic protocol for breast reconstructive surgery after mastoplasty in patients who have undergone breast carcinoma removal. This validation also may help further protocols as part of the treatment pathway for applications other than plastic and reconstructive surgery, such as infiltrative treatment for joint osteoarthritis, proctology, wound care, treatment of peripheral arterial disease, and chronic limb ischemia.

Our preliminary results showed that the adipose tissue maintains unaltered its cell population, as demonstrated by the adipocyte count of the viable cell population, whose trend exhibited a decrease over time (6 to 12 months), but without statistically significant differences. More interestingly, the cell viability assessed by MTT remained acceptable and constant up to 6 months with a slight decline (not statistically significant) at 12 months, with preservation of regenerative properties over time. These data also were confirmed by qualitative evaluation with the LIVE/DEAD test and by histological evaluation, showing no significant morphological or structural changes between the fresh tissue and thawed tissues at different time points. Finally, we assessed a panel of standard markers expressed by progenitor undifferentiated cells on fresh and thawed adipose tissues, again observing no qualitative differences between the investigated conditions.

In conclusion, we have demonstrated that a standardized and patented methodology allows cryopreservation of autologous lipoaspirate in a national authorized tissue bank for a long time, making available fat graft equivalent to fresh isolated tissue for patients who need multiple lipofilling procedures, thereby avoiding repeated liposuctions and related disadvantages.

There were some limitations to our investigation, including the relatively small sample size, and lack of analysis of the regenerative potency of the SVF component through quantitative immunohistochemical analysis. Moreover, more consolidated long-term data are required. From a clinical and surgical point of view, we will have to direct our endeavors to improving existing service to hospital centers that perform breast reconstructive surgery, including outpatient surgery, by offering opportunities to cryopreserve tissue on site, reducing the graft delivery time and ensuring maximum regenerative power of the tissue, and consequently the best clinical outcome.

CONCLUSIONS

In our preliminary in vitro findings, we describe an innovative protocol for collection, processing, cryopreservation, and thawing of autologous adipose tissue for multiple lipofilling surgical treatments for different purposes, which retains unchanged tissue morphology and acceptable adipocyte concentration when compared with fresh adipose tissue, and therefore optimal functional properties. Nevertheless, this cryopreservation and thawing protocol will be the subject of continued implementation, characterization, and standardization, addressing potential applications to surgical fields outside plastic and reconstructive surgery.

Supplemental Material

This article contains supplemental material located online at https://doi.org/10.1093/asj/sjae192.

Disclosures

Dr Ventura is co-author of the LipoBank S.r.l. Patent (EP 3994984 A1). Ms Di Fede is a clinical data manager consultant for LipoBank (Milano, Italy). Dr Alessandro Nanni-Costa is a scientific consultant for LipoBank.

Funding

The authors received no financial support for the research, authorship, and publication of this article.

REFERENCES

1. Strem BM, Hicok KC, Zhu M et al. Multipotential differentiation of adipose tissue-derived stem cells. *Keio J Med* 2005;54(3):132-141. doi: 10.2302/kjm.54.132

2. Schaffler A, Buchler C. Concise review: adipose tissue-derived stromal cells—basic and clinical implications for novel cell-based therapies. *Stem Cells* 2007;25:818-827. doi: 10.1634/stemcells.2006-0589
3. Krawczenko A, Klimczak A. Adipose tissue-derived mesenchymal stem/stromal cells and their contribution to angiogenic processes in tissue regeneration. *Int J Mol Sci* 2022;23(5):2425. doi: 10.3390/ijms23052425
4. Lee RH, Kim B, Choi I et al. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem* 2004;14(4-6):311-324. doi: 10.1159/000080341
5. Astori G, Vignati F, Bardelli S et al. In vitro" and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. *J Transl Med* 2007;5(1):55. doi: 10.1186/1479-5876-5-55
6. Kling RE, Mehrara BJ, Pusic AL et al. Trends in autologous fat grafting to the breast: a national survey of the American Society of Plastic Surgeons. *Plast Reconstr Surg* 2013;132(1):35-46. doi: 10.1097/PRS.0b013e318290fad1
7. Kaufman MR, Miller TA, Huang C et al. Autologous fat transfer for facial recontouring: is there science behind the art? *Plast Reconstr Surg* 2007;119(7): 2287-2296. doi: 10.1097/01.prs.0000260712.44089.e7
8. Wetterau M, Szpalski C, Hazen A, Warren SM. Autologous fat grafting and facial reconstruction. *J Craniofac Surg* 2012;23(1):315-318. doi: 10.1097/SCS.0b013e318241e1de
9. Tapp H, Hanley EN Jr, Patt JC, Gruber HE. Adipose-derived stem cells: characterization and current application in orthopaedic tissue repair. *Exp Biol Med* 2009;234(1):1-9. doi: 10.3181/0805/MR-170
10. Zhang Z, Yang X, Cao X, Qin A, Zhao J. Current applications of adipose-derived mesenchymal stem cells in bone repair and regeneration: a review of cell experiments, animal models, and clinical trials. *Front Bioeng Biotechnol* 2022;10:942128. doi: 10.3389/fbioe.2022.942128.
11. Freitag J, Li D, Wickham J, Shah K, Tenen A. Effect of autologous adipose-derived mesenchymal stem cell therapy in the treatment of a post-traumatic chondral defect of the knee. *BMJ Case Rep* 2017;2017:bcr2017220852. doi: 10.1136/bcr-2017-220852
12. Zhou L, Wang H, Yao S, Li L, Kuang X. Efficacy of human adipose derived mesenchymal stem cells in promoting skin wound healing. *J Healthc Eng* 2022;24: 6590025. doi: 10.1155/2022/6590025
13. Zollino I, Campioni D, Sibilla MG, Tessari M, Malagoni AM, Zamboni P. A phase II randomized clinical trial for the treatment of recalcitrant chronic leg ulcers using centrifuged adipose tissue containing progenitor cells. *Cytotherapy* 2019;21(2):200-211. doi: 10.1016/j.jcyt.2018.10.012
14. Masłowski L, Paprocka M, Czyżewska-Buczyńska A et al. Autotransplantation of the adipose tissue-derived mesenchymal stromal cells in therapy of venous stasis ulcers. *Arch Immunol Ther Exp (Warsz)* 2020;68(1):5. doi: 10.1007/s00005-020-00571-9
15. Katagiri T, Kondo K, Shibata R et al. Therapeutic angiogenesis using autologous adipose-derived regenerative cells in patients with critical limb ischaemia in Japan: a clinical pilot study. *Sci Rep* 2020;29; 10(1):16045. doi: 10.1038/s41598-020-73096-y
16. Smith OJ, Leigh R, Kanapathy M et al. Fat grafting and platelet-rich plasma for the treatment of diabetic foot ulcers: a feasibility-randomised controlled trial. *Int Wound J* 2020;17(6):1578-1594. doi: 10.1111/iwj.13433
17. Luck J, Smith OJ, Malik D, Mosahebi A. Protocol for a systematic review of autologous fat grafting for wound healing. *Syst Rev* 2018;7(1):99. doi: 10.1186/s13643-018-0769-7
18. Buscail E, Le Cosquer G, Gross F et al. Adipose-derived stem cells in the treatment of perianal fistulas in Crohn's disease: rationale, clinical results and perspectives. *Int J Mol Sci* 2021;22(18):9967. doi: 10.3390/ijms22189967
19. Laureti S, Gionchetti P, Cappelli A et al. Refractory complex Crohn's perianal fistulas: a role for autologous microfragmented adipose tissue injection. *Inflamm Bowel Dis* 2020;26(2):321-330. doi: 10.1093/ibd/izz051
20. Panés J, Bouma G, Ferrante M et al. INSPECT: a retrospective study to evaluate long-term effectiveness and safety of darvadstrocel in patients with perianal fistulizing Crohn's disease treated in the ADMIRE-CD trial. *Inflamm Bowel Dis* 2022;28(11):1737-1745. doi: 10.1093/ibd/izab361
21. Bianchi F, Maioli M, Leonardi E et al. A new nonenzymatic method and device to obtain a fat tissue derivative highly enriched in pericyte-like elements by mild mechanical forces from human lipoaspirates. *Cell Transplant* 2013;22(11): 2063-2077. doi: 10.3727/096368912X65785
22. Cohen SR, Tiryaki T, Womack HA, Canikyan S, Schlaudraff KU, Scheflan M. Cellular optimization of nanofat: comparison of two Nanofat processing devices in terms of cell count and viability. *Aesthet Surg J Open Forum* 2019;1(4): ojz028. doi: 10.1093/asjof/ojz028
23. Hang-Fu L, Marmolya G, Feiglin DH. Liposuction fat-fillant implant for breast augmentation and reconstruction. *Aesthetic Plast Surg* 1995;19(5):427-437. doi: 10.1007/BF00453876
24. Coleman SR, Saboeiro AP. Fat grafting to the breast revisited: safety and efficacy. *Plast Reconstr Surg* 2007;119(3):775-785; discussion 786-7. doi: 10.1097/01.prs.0000252001.59162.c9.
25. Coleman SR, Saboeiro AP. Primary breast augmentation with fat grafting. *Clin Plast Surg* 2015;42(3):301-306. doi: 10.1016/j.cps.2015.03.010
26. Rigotti G, Marchi A, Micciolo R, Baroni G. Autologous fat grafting in breast cancer patients. *Breast* 2012;21(5):690. doi: 10.1016/j.breast.2012.06.002
27. Khouri RK, Rigotti G, Khouri RK Jr et al. Tissue-engineered breast reconstruction with Brava-assisted fat grafting: a 7-year, 488-patient, multicenter experience. *Plast Reconstr Surg* 2015;135(3):643-658. doi: 10.1097/PRS.0000000000001039
28. Khouri RK, Rigotti G, Cardoso E, Khouri RK Jr, Biggs TM. Megavolume autologous fat transfer: part II. Practice and techniques. *Plast Reconstr Surg* 2014;133(6):1369-1377. doi: 10.1097/PRS.0000000000000179
29. Munhoz AM, de Azevedo Marques Neto A, Maximiliano J. Optimizing surgical outcomes with small-volume silicone implants associated with autogenous fat grafting in primary and revision breast augmentation surgery: soft weight hybrid (SWEH) concept. *Aesthetic Plast Surg* 2022;46(3):1087-1103. doi: 10.1007/s00266-021-02653-1
30. Wederfoort JLM, Voeten N, Smeins NNPM et al. A Dutch randomized controlled study shows autologous fat transfer with pre-expansion is more cost-effective in the long term than implants. *J Plast Reconstr Aesthet Surg* 2023;84:398-412. doi: 10.1016/j.bjps.2023.06.01
31. Rigotti G, Marchi A, Stringhini P et al. Determining the oncological risk of autologous lipoaspirate grafting for post-mastectomy breast reconstruction. *Aesthetic Plast Surg* 2010;34(4):475-480. doi: 10.1007/s00266-010-9481-2
32. You JS, Chung YE, Baek SE, Chung SP, Kim MJ. Imaging findings of liposuction with an emphasis on postsurgical complications. *Korean J Radiol* 2015;16(6): 1197-1206. doi: 10.3348/kjr.2015.16.6.1197
33. Bayram Y, Sezgic M, Karakol P, Bozkurt M, Filinte GT. The use of autologous fat grafts in breast surgery: a literature review. *Arch Plast Surg* 2019;46(6): 498-510. doi: 10.5999/aps.2019.00416
34. Kim HY, Jung BK, Lew DH, Lee DW. Autologous fat graft in the reconstructed breast: fat absorption rate and safety based on sonographic identification. *Arch Plast Surg* 2014;41(6):740-747. doi: 10.5999/aps.2014.41.6.740
35. Yang HJ, Kang SY. Comparisons between fresh and cryopreserved fat injections in facial lipofilling. *Arch Craniofac Surg* 2020;21(1):15-21. doi: 10.7181/acfs.2019.00612
36. Shu Z, Gao D, Pu LLQ. Update on cryopreservation of adipose tissue and adipose-derived stem cells. *Clin Plast Surg* 2015;42(2):209-218. doi: 10.1016/j.cps.2014.12.001
37. Koçak P, Ünsal N, Canikyan S, Kul Y, Cohen SR, Tiryaki T. Comparison of adipocyte viability after short-term cryopreservation of adipose aspirates through 3 different techniques. *Aesthet Surg J Open Forum* 2023;5:ojad026. doi: 10.1093/asjof/ojad026
38. Devitt SM, Carter CM, Dierov R, Weiss S, Gersch RP, Percec I. Successful isolation of viable adipose-derived stem cells from human adipose tissue subject to long-term cryopreservation: positive implications for adult stem cell-based therapeutics in patients of advanced age. *Stem Cells Int* 2015;2015:146421. doi: 10.1155/2015/146421
39. Elliott GD, Wang S, Fuller BJ. Cryoprotectants: a review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology* 2017;76:74-91. doi: 10.1016/j.cryobiol.2017.04.004
40. Method for the cryopreservation of samples of adipose tissue. European Patent Application. EP 3 994 984 a1. 11.05.2022 bulletin 2022/1.
41. Thirumala S, Gimble JM, Devireddy RV. Evaluation of methylcellulose and dimethyl sulfoxide as the cryoprotectants in a serum-free freezing media for cryopreservation of adipose-derived adult stem cells. *Stem Cells Dev* 2010;19(4):513-522. doi: 10.1089/scd.2009.0173
42. Badowski MS, Muise A, Harris DT. Long-term biobanking of intact tissue from lipoaspirate. *J Clin Med* 2019;8(3):327. doi: 10.3390/jcm8030327
43. Irioda AC, Cassilha R, Zocche L et al. Human adipose-derived mesenchymal stem cells cryopreservation and thawing decrease α4-integrin expression. *Stem Cells Int* 2016;2016(1):2562718. doi: 10.1155/2016/2562718
44. Hwang SM, Lee JS, Kim HD, Jung YH, Kim HI. Comparison of the viability of cryopreserved fat tissue in accordance with the thawing temperature. *Arch Plast Surg* 2015;42(2):143-149. doi: 10.5999/aps.2015.42.2.143
45. Zhang PQ, Tan PC, Gao YM et al. The effect of glycerol as a cryoprotective agent in the cryopreservation of adipose tissue. *Stem Cell Res Ther* 2022;13(1):152. doi: 10.1186/s13287-022-02817-z
46. Crowley CA, Smith WPW, Seah KTM, Lim SK, Khan WS. Cryopreservation of human adipose tissues and adipose-derived stem cells with DMSO and/or trehalose: a systematic review. *Cells* 2021;10(7):1837. doi: 10.3390/cells10071837
47. Ohashi M. Cryopreserved fat: our clinical experience and applications. *Plast Aesthetic Res* 2020;7:26. doi: 10.20517/2347-9264.2020.1