Cryobiology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Cryopreservation of adipose tissue with and without cryoprotective agent addition for breast lipofilling: A cytological and histological study

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ARTICLE INFO

Keywords:
Freezing
Thawing
Adipose tissue
Adipocyte
Cryoprotectants
Dimethylsulfoxide (Me_2SO or DMSO)

ABSTRACT

In the second reconstructive phase of the breast after mastectomy, lipofilling is often necessary. Currently, lipofilling occurs immediately after autologous adipose tissue harvesting procedure, but most of the patients, usually, require multiple sessions to obtain a satisfactory result. Therefore, the need of repeated surgical harvesting outputs implies high risk of patients' morbidity and discomfort as well as increasing medical time and costs. The aim of our pilot study was to find out a feasible method to cryopreserve adipose tissue, in order to avoid reiterated liposuctions.

Lipoaspirates samples have been harvested from 10 women and preserved by three methods: (1) the first one, using 10% Me_2SO and 20% human albumin from human plasma as cryoprotective agents; (2) the second one, adding 5% Me_2SO as cryoprotective agent; 3) the last one, without any cryoprotective agent. Fresh and cryopreserved fat samples, obtained through the aforementioned processes, have been analyzed ex vivo. The efficiency of the cryopreservation methods used was determined by adipocyte viability and the expression of adipocytes surface markers.

Lipoaspirates stored at −196 °C for 3 months, after thawing, retained comparable adipocyte viability and histology to fresh tissue and no significant differences were found between the three methods used. Although the current results, differences between the methodologies in terms of viability may not become evident until breast lipofilling using frozen-thawed cryopreserved tissue.

1. Introduction

In post-mastectomy reconstruction, lipofilling [3] gives excellent outcomes in terms of qualitative and quantitative improvement of the surrounding tissues, even more after exposition to harmful insults such as radiotherapy. Although autologous adipose tissue transplantation has been optimized, its variable tissue resorption rate needs repeated surgery to gain the required mammary shape and volume. Given that the sampling phase must necessarily be carried out in the operating room, the aspirated adipose tissue can be implanted otherwise, after a light local anesthesia, in the outpatient setting. The possibility of collecting the adipose tissue needed, as required, for multiple implants in a single operating session without re-intervening on the patient, would bring significant advantages in terms of patients' decreasing of morbidity and of management costs' abatement of at least two-thirds.

Considering the significant advantages coming from cryopreserved adipose tissue's use, numerous studies have been recently carried out, with not unequivocal results of the engraftment of the tissue describing fat cysts as the only complication arising. However, they are of simple medical management.

Ohashi et al. [18] performed lipofilling for aesthetic purpose on 54 women who underwent breast augmentation using cryopreserved

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https://doi.org/10.1016/j.cryobiol.2021.07.016
Received 9 February 2021; Received in revised form 23 July 2021; Accepted 26 July 2021
Available online 30 July 2021

adipose tissue at −196 °C by slow freezing, adding a commercial cryoprotective solution without dimethyl sulfoxide (Me_2SO), resulting in a full patient satisfaction and absence of complications with a follow-up of about 2 years. Mashiko et al. [16] conducted a study about the biological properties and therapeutic values of cryopreserved adipose tissue. They removed the adipose tissue from six patients, cryopreserving these samples both with the same method as Ohashi et al. and by simply placing them at −80 °C, not adding any cryoprotective agent (CPA). They observed that the majority of the adipocytes in the control resulted as necrotic. Although Clover and Buckley [2], remarking the aforementioned study, believed that probably the cryopreservation method used should be perfected.

Reviewing the related literature, among the various non-permeable CPAs, the 10% solution of Me_2SO and fetal bovine serum (FBS) is the most used for the cryopreservation of large volumes of cells and tissues. However, Me_2SO is cytotoxic at temperatures over 4 °C [23]. The clinical use of frozen/thawed cells cryopreserved with 10% of Me_2SO have caused many adverse effects and toxic reactions such as respiratory depression and neurotoxicity [1,22]. Furthermore, the use of FBS in the cryoprotective solution should be minimized because of its ability to trigger the xenogenic immune response or transmit pathogens to the recipient [21]. Anyway, it can be replaced with a xeno-free component such as human albumin. The problem is that any kind of CPA has, even if minimal, a significant toxic negative influence on the cells.

Patients and surgeons high satisfaction rates maked lipofilling a best accessory in the setting of oncologic reconstruction following both breast-conserving therapy and mastectomy so the treatment is not for aesthetic reasons [14].

The aim of this pilot study was to find out a feasible procedure to cryopreserve adipose tissue, in order to avoid repetitive liposuctions in breast reconstruction after breast cancer. Specifically, the study pursues the analysis of taken and cryopreserved adipose tissue using three different Me2SO concentrations (10%, 5% and 0%) to evaluate its effect on biological viability.

2. Materials and methods

The study involved 10 patients undergoing lipofilling for breast reconstruction at the “Giovanni Paolo II” Bari Oncological Institute. Patients with acute complications caused by actinic damage, such as radiodermatitis, ulcers and skin necrosis; diabetic patients; patients taking oral anticoagulant therapy; patients with a BMI of less than 19 had been excluded. The Ethics Committee of “Giovanni Paolo II” Oncological Institute had approved the research protocol before the study began (Prot. N754/2019).

2.1. Harvesting

Coleman’s technique [3] had been used for fat harvesting, in order to minimize any possible adipocytes’ trauma. With a 3-mm, blunt-edged, 2-hole cannula connected to a 10-mL syringe, fat had been manually suctioned by withdrawing the plunger. The cannula had been pushed through the harvest site, as the surgeon uses digital manipulation to pull back on the plunger of the syringe and create a gentle negative pressure. The collected fat samples had been spun at 3000 rpm for 3 min. The centrifuged adipose tissue had been deprived of the supernatant and the blood phase, preserving the intermediate layer consisting of viable fat cells. Finally, the purified graft obtained had been transferred to 8 3 mL syringes with the aid of a two or three-way tap. The procedure did not involve the infiltration of Klein solution, a tumescent anesthesia that uses large volumes of saline with local anesthetic (usually lidocaine 500–1500 mg/l) and epinephrine (0.5–1.5 mg/l) [9,12], in order to avoid the alteration of adipocyte membranes’ osmotic balance.

2.2. Cryopreservation

The lipofilling usually is done with small syringes to both ensure minimal necrosis and to provide more control and less pressure to deliver aliquots [15]. Sampling was accomplished with the aid of sterile 3-ml syringes. Three different procedures had been performed: 2.5 mL of the adipose tissue syringe’s contents had been placed in a sterile 5 mL cryogenic tube (cat no. CL5ARBIPSTS; Biosigma, Cona, Italy) and mixed with different cryoprotective solutions in each of which DMEM-HAM’S F-12 (cat no. ECM0090L; Euroclone, Pero, Italy) had been used as the base medium:

1) 10% Me_2SO Cell culture grade (cat no. EMR385050; Euroclone) and 20% human albumin from human plasma (Albumina Grifols 200 g/l; Instituto Grifols, Barcelona, Spain);
2) 5% Me_2SO;
3) no CPA.

Within 10 min the tubes had been frozen in a program-controlled-rate freezer (Planer Kryo 560–16, Planer, Middlesex, UK) at a slow cooling rate (−1 °C/min) to −80 °C. After one night at −80 °C, the cryogenic tube had been stored at the vapor phase of liquid nitrogen in a storage dewar (−196 °C) in the Institutional Bio-Bank of the IRCCS “Giovanni Paolo II” Oncological Institute (Bari, Italy). After 3 months of cryopreservation, the dewar cryogenic tube had been taken, and quickly thawed using a thermostatic bath with stirring at 37 °C. The content had been washed 2 times in PBS and centrifuged at 300×*g* for 5 min at environmental temperature to remove the CPA.

2.3. Histochemical and immunohistochemical analysis

Both fresh and thawed adipose tissue had been fixed in formalin, paraffin-embedded, hematoxylin-eosin–stained or/and incubated with the anti-Perilipin-1 antibody (Rabbit poyclonal to Perilipin-1; 1:200 dilution; cat no. ab3526; Abcam, Cambridge, UK) to evaluate the adipocytes’ morphology and viability. Sections had been deprived from paraffin by Clearify Clearing Agent, rehydrated, and antigen-retrieved by Dako 3 in 1 AR buffer citrate pH 9 in a DAKO PT link. Slides had been incubated with primary antibody anti-Perilipin-1 for 20 min at 32 °C, and peroxidase blocked with En FLEX Peroxidase-Blocking Reagent (a hydrogen peroxide, phosphoric acid, monosodium salt, monohydrate solution) for 3 min. They had been detected by Dako Envision Flex Rabbit LINKER and amplificated with kit EnV FLEX/HRP for 20 min. Colorimetric detection had been completed adding diaminobenzidine for 5 min. Slides had been counterstained using hematoxylin, and cover-lipped under DePeX. They had been acquired through a D-Sight slide scanner and high-resolution images had been analyzed by D-Sight Viewer software (A. Menarini Diagnostics, Firenze, Italy) in order to detect and count perilipin-positive cells (viable adipocytes).

2.4. Adipocyte count and phenotype

The fresh and thawed adipose tissue had been enzymatically digested using an adipose tissue dissociation kit (cat no. 130-105-808; Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer’s protocol, for 20 min at 37 °C under continuous agitation, using the MACS mix Tube Rotator (Miltenyi Biotech). After digestion, PBS had been added and mixed all together by inversion. The mixture had been centrifuged at 150×*g* for 10 min at room temperature (to prevent adipocytes’ mechanical damage), and the adipocyte rich layer immediately below the floating lipid layer had been collected. This cell suspension had been incubated at 4 °C in the dark for 10 min using the APC-conjugated anti-human CD36 antibody (clone REA760, IgG1; Miltenyi Biotech), the PE-conjugated anti-human CD45 (clone REA747, IgG1; Miltenyi Biotech) antibody, with 1 μL of BODIPY 493/503 dye (Thermo Fisher Scientific, Waltham, MA, USA) solution (20 μg/mL in ethanol)

and by 7-amino-actinomycin D (7-AAD Viability Dye; Beckman Coulter, Miami, USA), immediately followed by flow cytometric analyses through a NAVIOS flow cytometer (Beckman Coulter, Miami, Florida).

2.5. Statistical analysis

Statistical analysis had been conducted using GraphPad Prism software (GraphPad Software, San Diego, USA). Percentage cellular viability was expressed as mean ± standard deviation (SD). Data had been analyzed by one-way analysis of variance (ANOVA), and differences between groups had been assessed through Tukey's *post hoc* multiple comparisons test. A P-value of <0.05 had been considered to be statistically significant.

3. Results

3.1. Microscopic examination

Hematoxylin-eosin staining revealed adipose tissue sections preserve their morphological characteristics as cells delimited by evident cytoplasmic membrane circumscribing an optically empty cytoplasm, where the existing lipid droplets are dissolved during the usual tissue processing steps (Fig. 1, upper line). In a peripheral position, placed on the inner edge of the cytoplasmic membrane, the nucleus is highlighted, in a rounded or, sometimes, elongated shape, occasionally provided with a small subnuclear vacuole. Among the adipocytes it is possible to note the presence of small and medium caliber capillary vascular structures, and bundles of collagen fibers. Most of the frozen-thawed tissues highlighted adipocyte regressive phenomena present in the fresh unfrozen tissues, too, as a minimal percentage.

3.2. Immunohistochemical evaluation

Perilipin-1 modulates adipocyte lipid metabolism. It coats lipid storage droplets to protect them from hormone sensitive lipases (HSL) degradation. Perilipin-1 lack can cause thinness. Immunohistochemical images on adipose tissue against Perilipin-1 (Fig. 1, lower line) allowed to highlight not only the adipocyte morphological integrity through an intense membrane reaction (dark brown color), but even their cell viability since, their lack, would have shown their degradation by lipases. The percentage of positivity to perilipin-1 of the single adipocytes was remarkably high in 10% Me_2SO/20% albumin (97.66 ± 0.92%) (Fig. 1B), in 5% Me_2SO (95.24 ± 1.49%) (Fig. 1C), as well as in cryopreserved without CPA (96.47 ± 1.09%) (Fig. 1D), similarly in those fresh control group (93.26 ± 3.03%) (Fig. 1A). The respective quantitative analysis for proportion of perilipin-positive adipocytes is shown in Fig. 1.

3.3. Cryopreservation effects on adipocyte viability

A panel of markers had been analyzed to study the adipocyte phenotype from fresh and frozen-thawed adipose tissue. A dot plot depicting side scatter time of flight (SS-TOF) versus SS peak had been first used to gate single cells. Aggregates, which escaped the single cell gate had been observed as the few events high in FS-TOF signal in the dot plot (Fig. 2A). Debris and auto-fluorescence had been removed by using forward scatter (FS) INT versus SS INT (Fig. 2B). CD36 antibody binding had been used by BODIPY dying to collect high fluorescence cells (adipocytes) (Fig. 2C), also removing leukocyte contamination, CD45 negative adipocytes (Fig. 2D). Next, by employing staining with 7-AAD, a dye that is excluded by living cells, we had been able to distinguished living adipocytes from dead ones (Fig. 2E).

At the same time, we demonstrated that 99.39 ± 0.32% of fresh adipocytes resulted as negative for staining with 7-AAD dye, comparable to CPA-free freezing adipocytes (98.80 ± 0.57%). Flow analyses of frozen-thawed adipocytes exposed to 10% Me_2SO + 20% albumin (99.14 ± 0.69%) and 5% Me_2SO (97.77 ± 2.59%) in the cryoprotective solution revealed no differences in viability (Fig. 2F). All this showing that the adipocyte viability between fresh and frozen-thawed adipose tissue had been comparable, whether or not using these CPAs.

4. Discussion

In recent years, breast lipofilling has been performed widely in breast cancer surgery. At breast's level, anyway post-mastectomy reconstruction is carried out, even more, after exposure to harmful insults such as radiotherapy and chemotherapy, excellent results are gained in terms of qualitative and quantitative improvement regarding both the softness and the volume of the treated tissues, containing and reducing the possible complications described in the literature. Autologous adipose tissue is considered to be an ideal filler material because it causes fewer adverse reactions in comparison to other fillers such as hyaluronic acid, calcium hydroxylapatite, polyalkylimide, poly-L-lactic acid, polymethylmethacrylate microspheres [10,19]. Furthermore, since using the patient's own tissue, it is biocompatible. Harvesting fat through liposuction is a comfortable, repeatable, and low-cost procedure for most patients [3].

However, the main challenge of breast lipofilling is the variable rate of absorption at the grafted site. The fat reabsorption rate ranges from 25% to 52% [11]. These are not inherent to the transfer itself, but to factors that affect the collection, processing and injection of fat. These rates are believed to be related to both reduced cell viability after stress associated with adipose tissue harvest and a lack of transplant angiogenesis [5,13]. Eto et al. reported that one cause of high absorption rate is the lack of microcirculation, which can result in hypoxia, adipocyte apoptosis, necrosis, and fat liquidization [4]. Prior to revascularization, the graft fragments receive oxygen and glucose from the surrounding tissues. However, passive diffusion is inadequate to provide the required oxygen and glucose supply to adipocytes located in the center of the graft fragments. Therefore, in order to increase the survival rate of fat, it is important to ensure that these adipocytes, which are located between the highly perfused outer cell layer and the anoxic central core, are not subject to the apoptotic pathway [20].

Considering that in each session only small quantities of fat are able to graft to take root properly, lipofilling needs to be repeated several times (generally at least three months apart). Therefore, the need of repeated surgical harvesting outputs implies high risk of patients' morbidity and discomfort as well as increasing medical time and costs. The fat left over after the breast lipofilling is generally discarded, and that prompted us to explore ways to use it, in order to perform through just one liposuction multiple lipofilling.

Adipose tissue consists predominantly of mature adipocytes, and the viability variation of these cells and adipose tissue post freeze/thaw stress has implications for autologous fat graft performance in plastic surgery. Intracellular and extracellular ice development and osmolality disequilibrium will arise depending on the type of CPA used [17]. Many authors describe different ways to cryopreserve adipose tissue but all of them imply a CPA addition and we know that there can be severe neurotoxicity reaction to them [1,21–23]. The CPA Me_2SO is able to rapidly diffuse into the cell through the plasma membrane, reducing the number and size of intracellular ice crystals that would otherwise damage the membrane and cell organelles and protecting cells from dehydration. Due to the liquified and floating nature of fat samples, despite a double wash, a minimal amount of Me_2SO still remained and this could potentially be toxic [7] if this tissue is grafted.

In this pilot study, we present an analysis of our ex vivo results with a comparison between fresh and cryopreserved adipose tissue by three methods: (1) the first one, using 10% Me_2SO and 20% human albumin from human plasma as CPAs; (2) the second one, adding 5% Me_2SO as CPA; 3) the last one, without any cryoprotective agent.

Since hematoxylin and eosin staining cannot, with certainty, distinguish dead adipocytes from living ones, we have used, as a sensitive and

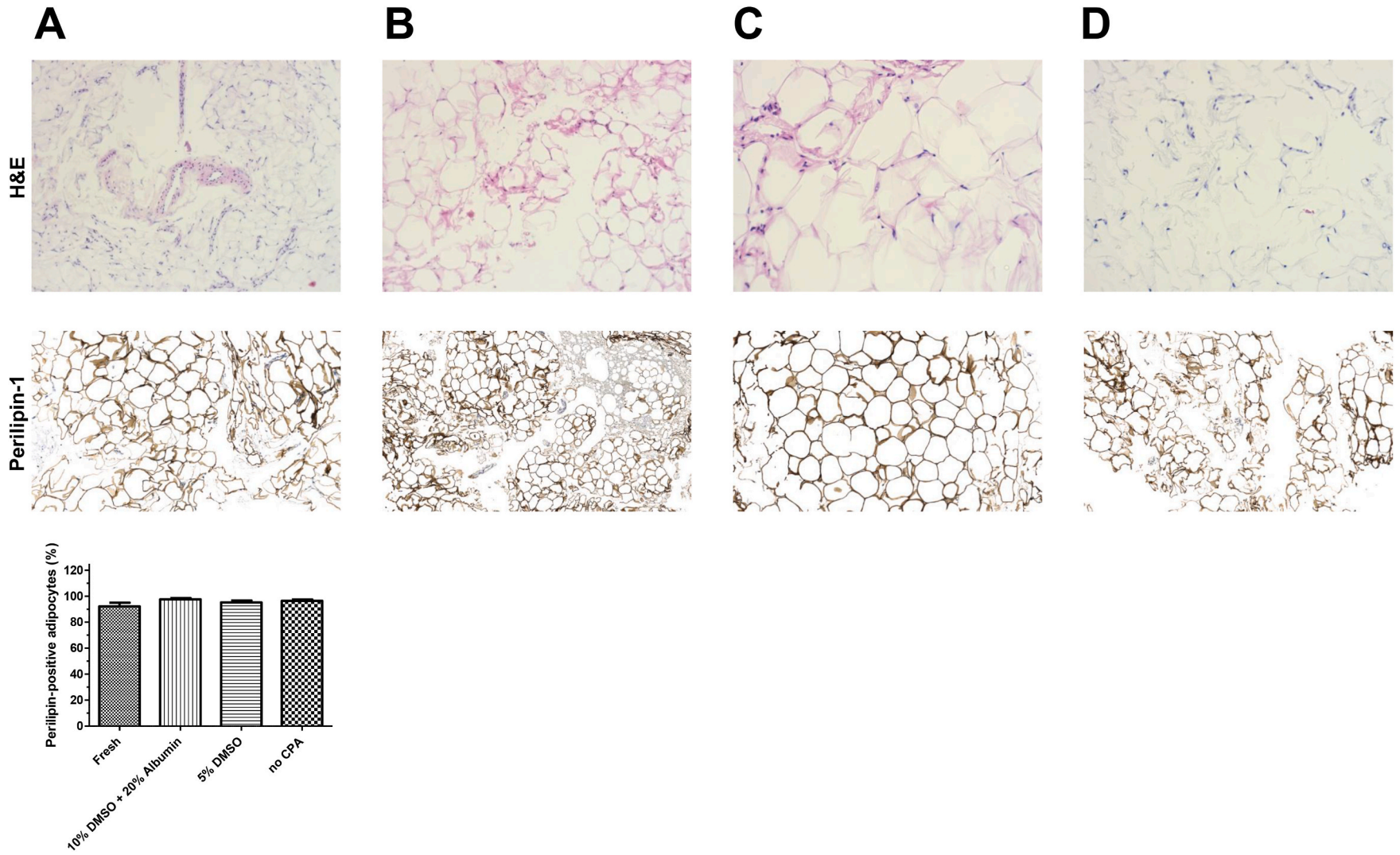

Fig. 1. (Upper line): Representative images on fresh adipose tissue (magnification 10x) (A), frozen-thawed with Me_2SO 10% CPA (magnification 10x) (B), with Me_2SO 5% (magnification 20x) (C) and without CPA (magnification 20x) (D) after formalin fixation, paraffin embedding and hematoxylin-eosin staining. (lower line): Representative images (magnification 10x) of adipocytes where perilipin-1 signal is shown as dark brown on fresh adipose tissue (A), frozen-thawed with Me_2SO 10% CPA (B), with Me_2SO 5% (C) and without CPA (D) after formalin fixation and paraffin embedding. (below): The bar chart shows mean ± SD of the adipocyte viability rate (%) in adipose tissue in the four different groups from counting results for perilipin-1 positive adipocytes; ten fields for every section of the 10 patients were counted. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

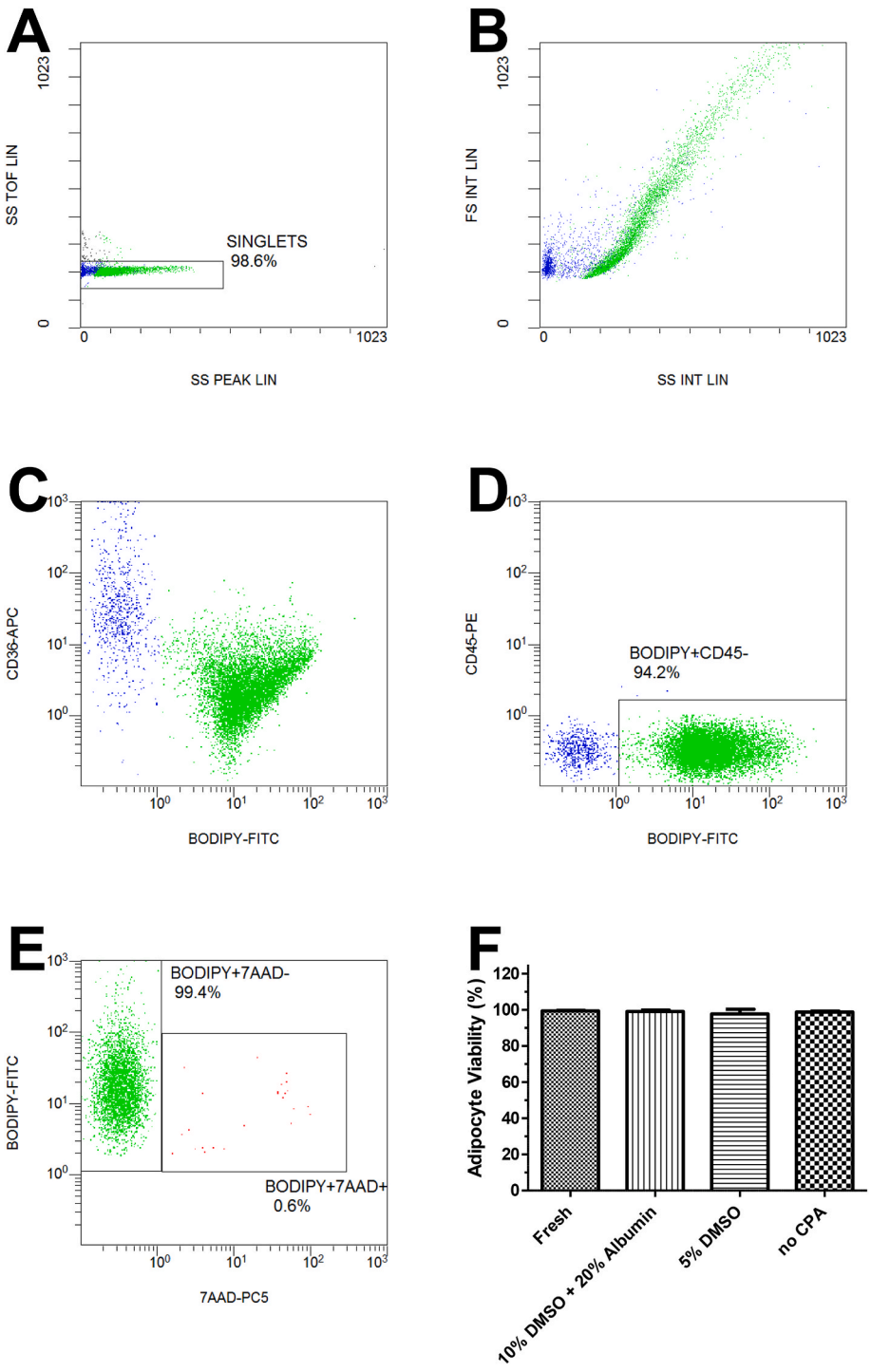

Fig. 2. Representative flow cytometric analysis of viability of adipocytes. (A) SS TOF LIN vs SS PEACK LIN dot plot for doublets exclusion, gate on singlets. (B) FSC vs SSC dot plot showing physical characteristics of adipocytes (green). (C–D) Dot plot showing events for the three fluorescence signals: adipocytes are identified as $CD45^{-}$ $CD36^{+}$ BODIPY + events; the events $CD45^{-}$ $CD36^{+}$ BODIPY- (blue) identify erythrocytes. (E) Dot plot showing viable adipocytes identified as BODIPY+ 7AAD-; dead adipocytes are identified as BODIPY+ 7AAD+. (F): The bar chart indicates the percentage ratio (mean ± SD) (n = 10) of adipocyte viability in the four different groups calculated from flow cytometric results, revealing no changes in adipocyte viability. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reliable indicator of adipocyte viability, perilipin-1 immunohistochemistry which easily identifies dead adipocytes where perilipin expression is lost [4,16,24]. This study showed that frozen-thawed adipose tissues was almost all perilipin-positive with no significant difference between cryoprotective solutions used and were not affected by Me_2SO concentration.

Furthermore, combining a lipid specific fluorescent stain (BODIPY) [8] with CD36 adipocyte cell surface marker [6], we observed that the presence of Me_2SO had a neutral effect on the adipocyte viability rates.

The main limitation of this short-term pilot study is that we have only reported ex vivo measures of viability which may not reflect the relative efficiency of the cryoprotective solutions tested after lipofilling. An additional in vivo study is needed. Another limitation is that we did not have tested adipocyte recovery because we considered as fundamental adipocytes' overall volume since lipofilling is used to restore loss of volume due to mastectomy or breast-conserving treatment.

Our data would support that the use of a cryoprotective solution without CPA is equal to others mixed with CPA, and that by harvesting the amount of fat required, no additional liposuction is needed.

5. Conclusion

In conclusion, the present pilot study tested the efficiency of cryopreservation via a slow-freezing/rapid-thawing protocol of human

adipose tissue using three different cryoprotective solutions. The expression of adipocytes surface markers of adipose tissues frozen in different cryoprotective solutions remained unaffected in comparison to fresh adipose tissue, as well also the post thaw viability of adipose tissues was found to be similar across the various solutions. Considering our study and the above-mentioned ones, further validation studies including more patients will be necessary to investigate breast lipofilling of frozen-thawed cryopreserved tissue.

Author contribution statement

G.M. and G.D. planned the research, coordinated the study, designed and performed most experiments, analyzed the respective data and drafted the manuscript; A.N. carried out the flow cytometric experiments; F.M. and D.L. performed immunohistochemical experiments; A. V.P. participated in the coordination of the study and assisted in manuscript preparation; C.M.R. performed fat harvesting procedure and supervised the research.

Declaration of competing interest

None to declare.

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