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Optimization of Adipose Tissue Cryopreservation Techniques in a Murine Model

María Eloísa Villaverde-Doménech, PhD*†
Roberto Moltó-García, PhD‡
Virina Gonzalez-Alonso, MD§
Juan Pablo Aracil-Kessler, PhD*
Carmen Carda-Batalla, PhD¶
Edurne Novella-Maestre, PhD ‖**

Background: The aim of this study was to develop an adipose tissue (AT) cryopreservation protocol that is effective, simple, and maintains the functionality and viability of AT after thawing and transplantation.
Methods: Two cryopreservation temperatures (T°), −20°C and −80°C, and two cryoprotective agents (CPAs), trehalose and hydroxyethyl starch (HES), were compared first in an experimental study, using a slowfreezing protocol. The five experimental groups were the following: (a) Fresh AT (control group), (b) T = −20°C, 10%HES, (c) T = −80°C, 10%HES, (d) T = −20°C, 0.35M trehalose, (e) T = −80°C, 0.35M trehalose. We evaluated the morphology (histological studies) and tissue viability by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genic expression. Based on the results of the preliminary study, an in vivo study was performed, choosing as cryopreservation T° −20°C. HES and trehalose were compared as cryoprotective agents and with a control group (fresh AT). AT grafts were transplanted into immunodeficient mice. After 1 month of inoculation, animals were euthanized and samples were recovered. Samples were weighted and processed for histological study, viability study (GAPDH genic expression), and vascularization study (VEGF genic expression).
Results: The initial histological study demonstrated that all AT cryopreserved group samples showed typical histological features of AT, similar to that of the control group. Statistically significant differences were not observed ($P > 0.05$) in GAPDH expression between different groups related to temperature or CPA. Referring to the in vivo studies, cryopreserved groups showed good take of the graft and normal AT architectural preservation, as well as a clear vascular network. Statistically significant differences were not found ($P > 0.05$) with regard to graft take (%), GAPDH, or VEGF expression.
Conclusion: Slow freezing at −20°C using trehalose, and −20°C using HES as cryoprotective agents are both straightforward and easy AT cryopreservation procedures, with results similar to those of fresh AT in terms of tissue viability and morphohistological characteristics. *(Plast Reconstr Surg Glob Open 2021;9:e3926; doi: 10.1097/GOX.0000000000003926; Published online 11 November 2021.)*

*From the *Plastic and Reconstructive Surgery Service, Consorcio Hospital Provincial de Castellón, Castelló, Spain; †Clínica de Cirugía Plástica Dra. Villaverde, Valencia, Spain; ‡Clínica de Cirugía Plástica Moltó, Valencia, Spain; §Plastic and Reconstructive Surgery Service, Hospital Clínico Universitario de Valencia, Valencia, Spain; ¶Cátedra en Departamento de Histología de la Universitat de València; ‖ Unidad de Genética, Hospital Universitario y Politécnico La Fe, Valencia, Spain; and **Reproductive Medicine Research Group, Instituto de Investigación Sanitario La Fe, Valencia, Spain.*

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INTRODUCTION

Of all the progress made in the field of plastic and reconstructive surgery over the past 20 years, the use of adipose tissue (AT) especially stands out. This technique has numerous applications and is extremely important in breast cancer reconstruction. Indeed, it has been proven that AT can correct and prevent breast sequelae and retractions, which are both common side effects of oncological breast treatments.[1–4] AT grafting is a simple, low-cost, and versatile technique, with low associated morbidity rates. One of its main problems is that, because of variable rates of fat absorption, repeated procedures to achieve the desired result are required.[4,5]

However, repeated harvest procedures can increase the cost of treatment and surgical risks, as well as the associated

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pain and discomfort for the patient. Thus, the ability to cryopreserve AT harvested for grafting would be particularly useful in this field. Therefore, the aim of this present work was to optimize and simplify the currently available AT cryopreservation techniques and protocols, without decreasing the functionality of the tissue after thawing and transplantation. We aimed to validate a cryopreservation protocol used to preserve AT at low temperatures for its later use for reconstructive purposes in clinical contexts.

METHODS

This experimental study was performed in two stages. In the first stage, we compared the efficacy of the AT recovered after using a slow-freezing protocol with storage at one of the two different cryopreservation temperatures (−20°C or −80°C), either using trehalose or hydroxyethyl starch (HES) as the cryoprotective agent (CPA). After validating the cryopreservation protocols, in the second phase of the study, we performed an in vivo transplantation into immunodeficient mice to examine AT viability, engraftment, and vascularization.

For both phases of the study, we harvested samples from human patients. The human AT used had either been cryopreserved (experimental groups) or was freshly processed (control group). All the procedures were performed in accordance with the regulations on human experimentation set out in the Nuremberg code and Declaration of Helsinki (1964). The experimental study was approved by our institutional review board (the Ethics Committee for Biomedical Research at the Hospital Universitari I Politècnic La Fe), while the animal experiments were authorized by the Spanish Animal Health and Welfare Service of the General Directorate of Agricultural Production and Livestock.

Preliminary Study

Study Participants

Men or women aged 18–60 years with no systemic disease or active tumors were considered for inclusion, while individuals with a previous history of cytostatic, corticoid, or immunosuppressive treatment were excluded. The preliminary study included four patients.

Sample Harvest

Tumescent anesthesia was achieved by infiltrating in the abdomen and anterior thighs of the donors with modified Klein solution (1 cm^3 of modified Klein solution per 1 cm^3 of AT to be extracted). Modified Klein solution was obtained by diluting 1 mL of adrenaline (1 mg/mL; B. Braun, Melsungen, Germany) in 500 mL of Ringer lactate solution (Viaflo Hartmann; Baxter, Deerfield, Ill.), at a final concentration of 1:500,000. For procedures performed under local anesthesia, 10 mL of 5% lidocaine (B. Braun) was also added to the solution.

The AT was aspirated from the donors in an atraumatic fashion with a 10 cm^3 Luer-lock syringe (Zarys, Zabrze, Poland), connected to a 3 mm diameter blunt cannula (Edipec, Barcelona, Spain) with two distal holes. The extracted AT was transferred using a Lipopras kit (Proteal, Barcelona, Spain) to 20 mL collectors designed for use in Duografter II centrifuges (Proteal). All the sample processing was carried out in a laminar flow hood to avoid contamination. The samples were centrifuged at 3000 rpm for 1 minute, followed by separation of the middle AT layer from the other phases (serum and oil), following the technique described by Coleman.[2,6–8]

Sample Processing and Freezing

The ATs obtained from all the patients were pooled, then processed and frozen either at −20°C or −80°C following a slow-freezing protocol and using either trehalose or HES as the CPA. Briefly, the middle phase of the centrifuged sample was transferred to ice and was then divided into five equal parts for use in the following experimental groups: group 1, −20°C (Dulbecco modified Eagle medium [DEMEM]/F2 nutrient mixture medium[F2], with 10% HES); group 2, −80°C (DEMEM/F2, with 10% HES); group 3, −20°C (DMEM/F2, with 0.35 M trehalose); group 4, −80°C (DMEM/F2, with 0.35 M trehalose), and finally, fresh AT used as a control group. Thus, AT from all four preliminary study patients was present in all the groups. All the tissue manipulation and processing were carried out in a laminar flow hood in a controlled and sterile environment.

All the experimental groups were cryopreserved and mixed with the CPA by mixing 15 mL of AT and 15 mL of the appropriate CPA solution (trehalose or HES) in 50 mL Falcon tubes (I.C.T., Lardero, La Rioja, Spain). The mixture was then divided into different 2 mL cryogenic vials (Fisher Scientific, Madrid, Spain) and kept at room temperature for 5 minutes. The samples in groups 1–4 were frozen to −20°C by placing them in a commercial container (Antech Scientific, Qingdao, China) with a cooling speed of −1°C/minute, so that they reached −20°C over 20 minutes. Subsequently, the samples assigned to groups 2 and 4 were placed directly into a specialized freezer maintained at −80°C (Liebherr, Bulle, Switzerland). Thus, the overall cryopreservation protocol took around 30 minutes. The control group was directly processed for use in morphological and viability studies, without freezing or thawing.

Thawing

One week after cryopreservation (groups 1–4), the samples were thawed by immersing them in a shacking water bath preheated to 40°C (Thermo Scientific, Waltham, Mass.). We then applied a dilution protocol in which 50% of the corresponding CPA solution was initially added to each sample at room temperature. Washes were subsequently carried out with decreasing CPA concentrations (25% and then 12.5%) and finally, the AT was washed three times in DEMEM/F2 culture media (Sigma, Barcelona, Spain). Each step took approximately 5 minutes, with the AT being kept in incubators at 37°C with 5% CO_2 in between each step. The samples were then processed for viability assessment and histological analysis.

Morphological Studies

Samples were fixed in 4% formaldehyde and washed and dehydrated with serial ethanol solutions, which gradually increased in concentration (50%, 70%, 80%, 90%; with three final washes in 100% ethanol). Next, the

samples were lightened and included in paraffin blocks. To try to increase the sensitivity of this work, we took 5-µm serial sections at different levels of the blocks. The sections were stained with hematoxylin–eosin to evaluate the histological characteristics of the samples.

Viability Study

To evaluate any possible tissue degeneration that fixers and cryopreservation conditions could have exerted upon the AT, and to carry out a preliminary estimation of the cryopreserved tissue viability, we determined the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene relative expression (ΔCt) by polymerase chain reaction (PCR) amplification. First, we extracted messenger ribonucleic acid (mRNA) from the samples and converted it into complementary deoxyribonucleic acid (cDNA) by reverse transcription. We then quantified the *GAPDH* expression by carrying out fluorescent real-time quantitative fluorescence PCR (QF-PCR) with TaqMan reagents. The development of the technique, its normalization, and quantification of results have been previously described elsewhere.[9,10]

GAPDH is a multifunctional protein that plays different roles according to its subcellular localization, oligomeric state, and interactions with other proteins or ligands.[11,12] Mammalian cellular processes involving GAPDH include transport, membrane fusion, microtubule assembly, transcriptional regulation, ribonucleic acid (RNA) exportation, deoxyribonucleic acid (DNA) replication and reparation, and apoptosis.[11–13] In addition, GAPDH is commonly used as a late adipocyte differentiation marker and as an adipocyte viability marker.[11–13]

In Vivo Study

After validating the cryopreservation protocols in our preliminary studies, we transplanted control AT or AT obtained using two of these protocols into a murine model. We also checked whether the cryopreservation time (1 or 2 months) influenced the engraftment efficacy and implant viability. The in vivo study employed the same methodology from the preliminary study, with the following variations: AT samples collected on the same day (day 0) from six patients not used in the preliminary work. The samples were pooled before transplantation; so AT from each donor was present in all the experimental groups.

As outlined in Figure 1, the following AT samples were transplanted on days 0, 30, and 60: fresh AT (control, transplanted on day 0); group A, –20°C (DEMEM/F2 with 10% HES), cryopreserved for 1 month (transplanted on day 30); group B, –20°C (DEMEM/F2 with 10% HES), cryopreserved for 2 months (transplanted on day 60); group C, –20°C (DMEM/F2 with 0.35 M trehalose), cryopreserved for 1 month (transplanted on day 30); group D, –20°C (DMEM/F2 with 0.35 M trehalose), cryopreserved for 2 months (transplanted on day 60).

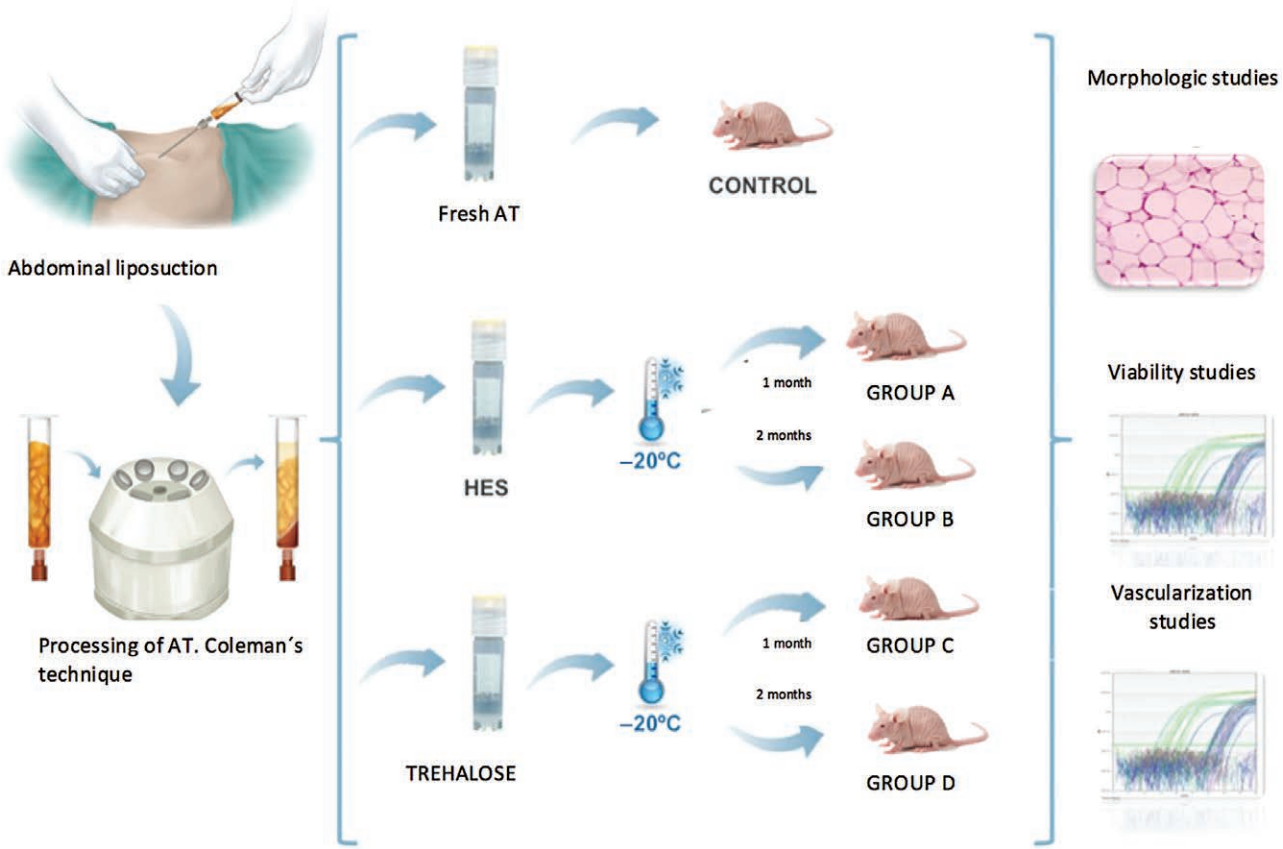

Fig. 1. Design of the in vivo study.

Xenotransplantation into Immunosuppressed Mice

In this study, we used 15 male Hsd:Athymic Nude-nu mice aged 6 weeks. The animals were kept in cages with a filter inside protective cubicles in a biosecurity level-2 installation. The environmental temperature was fixed at 21°C ± 1°C, with a light/darkness cycle of 12/12 h and a relative humidity of 55% ± 10%. The animals were fed ad libitum with irradiated feed and sterilized water and were manipulated inside a laminar flow cabinet.

Before injecting the AT, the mice were anaesthetized by inhalation of isoflurane through an anesthesia machine. Each AT sample was the same weight and volume; two separate samples were injected into in the subcutaneous tissue on the back of each mouse, using a 16-gauge blunt cannula, following a previously described protocol.[14,15] Thus, there was a final sample size of 30 implants, with two implants per mouse.

The animals were euthanized 1 month after the AT implantation by intraperitoneal injection of pentobarbital. The samples were then harvested, and the implants macroscopically analyzed by measuring their weight and volume, with their weight maintenance serving as a proxy for the AT implant engraftment efficacy (Fig. 2).

Morphological Studies

The samples were processed as described in our preliminary study, although we also characterized the implant engraftment and survival, vascular and nervous invasion, presence of fibrous areas, and vacuolization and inflammatory reactions (as nominal present/not present categorizations in each category). Samples were studied by optical microscopy (LEICA DM4000B; Leica, Solms, Germany) using a digital image capture system (Leica DFC450CM; Leica, Solms, Germany).

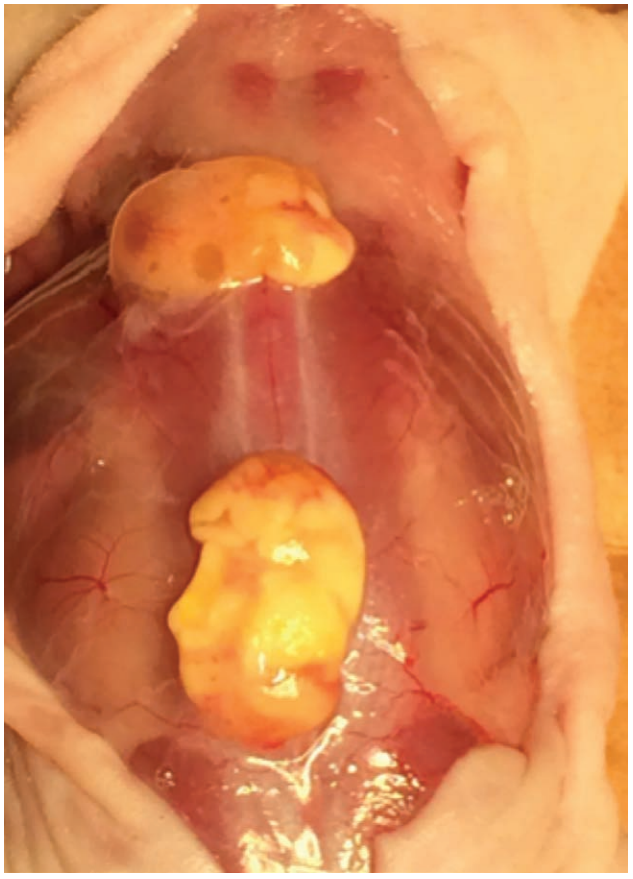

Fig. 2. AT grafts harvested 1-month postimplantation.

Vascularization Study

The same QF-PCR techniques and reagents used to study *GAPDH* relative expression levels were used to test the vascular endothelial growth factor (*VEGF*) gene relative expression in these in vivo studies. *VEGF* expression is an indicator of proper implant engraftment and vascular invasion and was therefore related to adequate AT integration into the recipient tissues.

Data Collection and Statistical Analysis

The data obtained were stored in a database, and statistical analysis was carried out using GraphPad InStat, v3.0 (San Diego, Calif.). The mean, SD, and standard error of the mean (SEM), were calculated, and the data are presented as the mean ± SD. After applying the Kruskal–Wallis test, nonparametric Mann–Whitney U tests were used to compare the control group values with the experimental groups. The Dunn multiple comparison test was applied as a post-test, applying Spearman–Brown correlation coefficients. Probabilities (*P* values) less than 0.05 were considered statistically significant.

RESULTS

Preliminary Study

Histomorphological Studies

The cellular architectures and structures were evaluated by studying the adipose cell conformation, cellular location within the extracellular matrix, positioning of the nucleus in each cell, and cell membrane integrity. The fresh AT structure and architecture was similar to that of all the experimental groups (HES or trehalose, frozen at −20°C or −80°C). (**See figure 1, Supplemental Digital Content 1,** which displays histomorphological study of the cryopreserved adipose tissue (AT). Control group: fresh AT, 20×; group 1 (G.1): cryopreserved AT with HES at −20°C, 10×; group 2 (G.2): cryopreserved AT with HES at −80°C, 10×; group 3 (G.3) cryopreserved AT with trehalose at −20°C, 10×, and group 4 (G.4), cryopreserved AT with trehalose at −80°C, 10×. **http://links.lww.com/PRSGO/B836**.)

Viability Studies

Conventional PCR showed that all the groups expressed similar levels of *GAPDH*; the ribosomal *18s* gene was used as a housekeeping control in these experiments. More sensitive and accurate Taq-Man QF-PCR tests also obtained similar expression values in the experimental groups (group 1 = 7.02 ± 2.33; group 2 = 9.02 ± 2.70; group 3 = 4.64 ± 0.91; and group 4 = 6.31 ± 0.65), with

no significant differences between these groups compared with the fresh tissue control (4.72 ± 0.13).

In Vivo Study

AT Implant Weight and Volume Evolution

The weight and volume of each sample were registered before implantation and after harvesting; maintenance of the implant weight indicated adequate AT engraftment. The average weights and volumes before implantation were 0.49 ± 0.19 and 0.48 ± 0.16, respectively; by the time of tissue harvesting, this had reduced to 0.13 ± 0.06 and 0.16 ± 0.07, respectively. Thus, the average implant engraftment was 26.61% ± 12.12%. This rate was highest in the control group at 33.77% ± 9.63% and was as follows for the experimental groups: group A = 18.88% ± 5.41%; group B = 32.17% ± 14.19%; group C = 22.1% ± 9.83%; group D = 26.09% ± 15.4%. No statistically significant differences were found between the experimental groups or compared with the control group.

Structure of Cryopreserved AT Implanted into a Murine Model

Histomorphological study of the cryopreserved AT showed that it had a proper configuration and structure, with signs of vascularization 1 month after implantation into the nude mice. These structural features were remarkably similar in both the experimental and fresh AT implants. (**See figure 2, Supplemental Digital Content 2,** which displays distomorphological analysis of the AT implants in the animal model. 4A and 4B: Fresh AT implants (4A: 40× and 4B: 10×). Observe the reinforcement connective tissue (4B, arrow). 4C and 4D: 1-month cryopreserved AT, 4C with HES (hydroxyethyl starch; 10×), 4D with trehalose (40×). Observe the vascular and nervous tissue invasion inside the AT implant (4D, asterisk). 4E and 4F: 2-month cryopreserved AT, 4E with HES (10×), 4F with trehalose (10×). The same findings were observed in terms of cellularity, histological conformation, and vascularization as in the control group and the 1-month cryopreserved group. **http://links.lww.com/PRSGO/B837**.)

Compared with the control group, we also observed proper conservation of the structure and cellularity when the AT had been cryopreserved for 1 month at −20 °C (with HES or trehalose) before transplant; the implant had been invaded by abundant vessels and nerves, denoting optimal engraftment (**SDC2**, **http://links.lww.com/PRSGO/B837**). The findings in terms of structure, configuration, cellularity, and vascularization were also similar for the AT that had been cryopreserved for 2 months (**SDC2**, **http://links.lww.com/PRSGO/B837**). To summarize, implant engraftment, survival, nervous and vascular invasion, presence of fibrous areas, vacuolization, and inflammatory reactions were comparable between the control and experimental groups.

Viability and Vascularization of Cryopreserved AT Implanted into a Murine Model

The viability and vascularization of the cryopreserved AT implants was studied by analyzing *GAPDH* and *VEGF* relative expression, using the ribosomal *18s* gene as a housekeeping control. *GAPDH* was expressed in all the samples (group A = 6.74 ± 0.43; group B = 6.54 ± 0.84; group C = 6.17 ± 0.17; group D = 7.26 ± 0.62; and control = 6.81 ± 0.63). *VEGF* was also expressed in all the AT samples (group A = 14.93 ± 1.94; group B = 14.27 ± 2.52; group C = 15.49 ± 1.61; group D = 16.61 ± 1.63; and control = 16.17 ± 1.72). No significant differences were found when comparing the *GAPDH* and *VEGF* relative expression in the different groups, or according to the different CPAs used, cryopreservation times, or when compared with the control group. No statistically significant differences ($P > 0.05$) were found for any of the comparisons.

DISCUSSION

We analyzed the weight maintenance as an approximation of the implant engraftment success and found that the average weight maintenance was 26.61% ± 12.12%, with the highest implant engraftment being in the control group (33.77% ± 9.63%) and group C (32.17% ± 14.19%). Nevertheless, no statistically significant differences in the implant engraftment were detected in relation to the use of different CPAs or cryopreservation times. This implant weight maintenance method is not commonly used in the studies related to cryopreservation techniques. However, Cui et al produced AT implant weight maintenance levels similar to those of ours in their control (44%) and an experimental group (32%) after AT implantation in immunosuppressed nude mice for 4 months.[15] AT implant engraftment is very variable, even in optimal conditions (ie, a clinical setting with fresh AT grafts with a compliant and healthy patient who is able to rest after the procedure, etc.), and so some degree of graft resorption always occurs. Thus, given that the implant engraftment was similar in all our groups (including the fresh tissue control), the moderate implant engraftment we detected could perhaps be explained by the constant activity of mice which would favor fat graft absorption.

Although the optimal temperature for the cryopreservation of human ATs is still under investigation, some studies suggest that −20°C is sufficient to maintain AT viability.[6,14,16] Moreover, compared with cryopreservation at −80°C or using liquid nitrogen, which would require a complex facility, extra space, and higher costs, freezing at −20°C is more feasible in a clinical context.[17] Li et al were unable to find any significant differences in cell viability between AT cryopreserved at −20°C, −80°C, or −196°C when using HES or a normal saline solution after injecting cryopreserved AT into nude mice for 2 and 4 weeks.[14] Furthermore, in 2000, Sommer et al used trypan blue staining—a method commonly used to assess cell viability[6,18]—to demonstrate that, slowly freezing AT to −20°C had no harmful effects on adipocyte viability, even when thawed 3 years after cryopreservation.[6] However, this method may overestimate AT cell viability given that cells with major damage, or without nuclear or mitochondrial activity, can still sometimes maintain a normal morphology under the microscope. Thus, trypan blue staining should be combined with other viability studies such as *GAPDH* analysis by PCR to assess AT viability.[19]

In the in vivo study, there were no statistically significant differences in cell viability (*GAPDH*) or vascularization (*VEGF*) marker relative gene expression between the samples cryopreserved for 1 versus 2 months. Most studies used AT cryopreserved for only hours or days,[15,20] although Atik et al cryopreserved AT for 6 months[17] and Chaput et al compared the results of cryopreservation after 8 versus 31 days at −80°C or −196°C in a CPA mixture of dimethyl sulfoxide and HES. These latter authors observed a nonsignificant decrease in cell viability after cryopreservation for 1 month, but significantly improved AT conservation at −80°C compared with storage at −196°C. Similarly, Choudhery et al found that the morphology, phenotypic marker expression, and proliferative potential of mesenchymal stem cells in cryopreserved AT were not compromised by cryopreservation.[21] This is significant because it indicates that cryopreserved AT could be used for regenerative medicine in the future.

What is clear in academic literature is the great heterogeneity between cryopreservation studies in terms of the temperatures, CPAs, cryopreservation and thawing methods, viability analyses, and methods used. Thus, in the absence of optimization of these parameters for AT, the ideal method for AT cryopreservation remains to be determined. However, cryopreservation using a slow-freezing protocol (decreasing by 1°C per minute) with a CPA, and rapid thawing in a water bath preheated to 37°C–40°C, does appear to help maintain good AT viability.[19] Here we aimed to develop a simplified cryopreservation protocol that could easily be applied in clinical contexts in the future. We used a slow-freezing and fast-thawing protocol, and tested cryopreservation efficacy with two nontoxic CPAs, both at −20°C and −80°C. We assessed numerous factors, including graft weight, volume, cell viability and graft revascularization, and morphological assessments to gain insight into the overall effects of these AT cryopreservation protocols, yielding valuable information and making this study more exhaustive than some previous work.

In summary, in our preliminary study, we did not find any differences at the histological level or in terms of *GAPDH* relative expression (as a proxy for cell viability) in AT cryopreserved at −20°C versus −80°C, or when using HES versus trehalose. Thus, in our murine model experiments, we froze the samples at −20°C because this would be easier to apply in the future in the context of clinical practice. Nevertheless, we cannot assume that in vitro studies are comparable to in vivo studies. Another limitation of this study was the small sample of only 15 athymic mice with 30 AT samples. However, in the absence of institutional funding, our budget from private funds limited the scope of this work. Nonetheless, the AT sample size we obtained was sufficient to obtain accurate determinations. To increase the statistical power of this work in the future, a bigger sample size would be required and may be able to detect significant differences in some of these comparisons. Furthermore, future studies should be randomized, and could analyze the effect of longer cryopreservation times or increased experimental animal implantation times to assess if there are any late resorption phenomena.

CONCLUSIONS

Cryopreservation of AT at −20°C or −80°C maintained similar viability and tissue survival rates compared with fresh AT. AT cryopreservation at −20°C using HES or trehalose was nontoxic and maintained good cell viability, allowing AT to properly engraft and vascularize in vivo after implantation in a vascularized recipient such as the athymic mice we used in this work. We showed that short-term cryopreservation (1–2 months) did not affect AT morphology, viability, or implant engraftment and vascularization in this experimental model. These cryopreservation protocols are simple and accessible techniques that, with additional studies performed in clinical settings, could be applied in clinical contexts in the near future.

María Eloísa Villaverde-Doménech, PhD
Plastic and Reconstructive Surgery Service
Consorcio Hospital Provincial de Castellón
Avenida Doctor Clarà, No. 19, 12002
Castelló de la Plana
Spain
E-mail: info@dravillaverde.com
Instagram: @doctora.villaverde
Facebook: Dra. Villaverde. Cirugía Plástica, Estética y Reconstructiva

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