Long-Term Preservation of Adipose Aspirates After Conventional Lipoplasty

Lee L. Q. Pu, MD, PhD; Xiangdong Cui, MD; Betsy F. Fink, BSc; Michael L. Cibull MD; and Dayong Gao, PhD

From the University of Kentucky, Lexington, KY. Dr. Pu is Assistant Professor, Division of Plastic Surgery, Department of Surgery, College of Medicine. Dr. Cui is a postdoctoral scholar in the Division of Plastic Surgery and the Department of Mechanical Engineering, College of Engineering. Ms. Fink is a research assistant in the Division of Plastic Surgery. Dr. Cibull is Professor, Department of Pathology, College of Medicine. Dr. Gao is Professor, Department of Mechanical Engineering, College of Engineering.

Background: *Optimal cryopreservation permits the long-term storage of living cells or tissues that may have potential clinical applications. Unfortunately, there are no successful studies on the long-term preservation of adipose aspirates for possible autologous fat grafting.*

Objective: *The purpose of the current study was (1) to test our hypothesis that adipose aspirates obtained from conventional lipoplasty could be preserved and stored at low temperature (below –85°C) by means of an optimal cryopreservation technique and (2) to develop a novel approach to effectively preserve adipose aspirates for future applications.*

Methods: *The middle layer of adipose aspirates obtained from conventional lipoplasty was collected after centrifugation and each specimen was then randomized into 3 groups: the control group, fresh adipose aspirates without preservation; experimental group 1, simple cryopreservation with liquid nitrogen only; and experimental group 2, optimal cryopreservation with cryoprotective agents consisting of a combination of dimethyl sulfoxide (DMSO) and trehalose. Cryopreservation of adipose aspirates was conducted with controlled slow cooling and fast rewarming rates. Fresh or cryopreserved adipose aspirates in each group were evaluated by viable adipocyte counts, glycerol-3-phosphate dehydrogenase (G3PDH) assay, and routine histology.*

Results: *Significantly more viable adipocytes and better cellular function of adipose aspirates were found in the experimental group 2 compared to the results in the experimental group 1.*

Conclusions: *Our results indicated that an optimal cryopreservation approach that utilizes a combination of DMSO and trehalose as cryoprotective agents appears to provide good long-term preservation of adipose aspirates obtained from conventional lipoplasty, albeit not as ideal as fresh specimens. An in vivo study will be conducted to confirm the results from our present in vitro study.* (Aesthetic Surg *J* 2004;24:536-541.)

Autologous fat transplantation (AFT) is a common cosmetic procedure performed by plastic surgeons to rejuvenate the face, hands, or other parts of the body secondary to aging. AFT has also been used extensively to correct irregularities caused by lipoplasty, for aesthetic improvement of body contour and to correct various soft tissue deficiencies of congenital, posttraumatic, or iatrogenic defects.[1] However, the main obstacle to achieving long-term favorable results of soft tissue augmentation after AFT is the high rate of absorption in the grafted site, which can reach up to 70% of the filled volume.[1,2] This high rate of absorption often necessitates either overcorrection or repeated procedures in the desired area, causing patient discomfort, less than optimal appearance, higher cost, and morbidity or trauma of the donor site.

At the present time, adipose aspirates can only be used for immediate autologous fat grafting at the time of extraction. Therefore, adipose aspirates obtained from lipoplasty are usually discarded. Until recently, few empirical experimental studies had been done to examine the role of frozen storage of adipose aspirates, and these involved only short-term preservation, at a relatively high temperature (+1°C to –18°C), of aspirates obtained by lipoplasty. However, these preliminary results were unsatisfactory.[3,4] Both plastic surgeons and patients have had a strong desire to preserve adipose aspirates obtained from conventional lipoplasty for potential future applications, assuming a reliable technique available for their long-term preservation.

The modern technique of cryopreservation permits the long-term storage of living cells and tissues that may

have many potential clinical applications, such as blood transfusion,[5] bone marrow transplantation,[6] in vitro fertilization,[7] vascular graft,[8] and bone graft.[9] The use of cryopreservation technology for long-term preservation of skin grafts and of nipples for autogenous reconstruction has been reported recently in the plastic surgery literature.[10,11] Unfortunately, there are no successful studies on the long-term preservation of autogenous fatty tissue for possible future transplantations.

It is our hypothesis that adipose aspirates obtained from conventional lipoplasty could be preserved and stored at low temperature (below –85 ºC) by means of an optimal cryopreservation technique. The purpose of the current study was to develop a novel approach to effectively preserve adipose aspirates after lipoplasty and to bank them for possible future AFTs. Such a novel approach would also be evaluated in vitro before future in vivo studies of autologous fat transplantation with cryopreserved adipose aspirates are undertaken.

Materials and Methods

Preparation of adipose aspirates

Adipose aspirates were obtained from specimens after conventional suction-assisted lipoplasty performed at University of Kentucky Medical Center and Lexington Healthsouth Outpatient Surgery center, both in Lexington, KY. The study was approved by the University's Institutional Review Board. All specimens were from the abdomens of 8 adult, white, female patients, aged from 26 to 56 years, who had no major systemic metabolic diseases or lipid disorders. The adipose aspirates (about 500 mL) were collected in a bottle at the time of lipoplasty and transferred immediately to the laboratory. The specimens were then spun at 50 *g* for 10 minutes on a large-capacity centrifuge (Mistral 3000i, Dearborn, MI) to separate adipose tissue from oil and soluble liquid. The middle layer of adipose aspirates after centrifugation, which contained more viable adipocytes, was taken for cryopreservation according to the experimental protocol. All procedures were performed at room temperature unless otherwise indicated.

Experimental protocol

Adipose aspirates from each patient were then randomized into 3 groups: the control group, fresh adipose aspirates without preservation; experimental group 1, simple cryopreservation of adipose aspirates with liquid nitrogen only; and experimental group 2, optimal cryopreservation of adipose aspirates with a combination of dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) and trehalose (Sigma, St. Louis, MO).

Cryopreservation technique

Choices of cryoprotective agents (CPAs). In this study, DMSO, a permeable CPA that could reduce cell injury due to the intracellular ice formation and "solution effects," and trehalose, a nonpermeable CPA that could protect the cell membrane, were selected as cryoprotective agents.[12,14] Many different concentrations and combinations of both CPAs were tried in the early stage of our experiment in order to find the most effective method of cryopreservation for adipose aspirates. A combination consisting of 0.5 M (3.3%) DMSO and 0.2 M (7.6%) trehalose was determined to be best and was used later in this study. Double concentrations of the different CPA solutions were made first and each chosen CPA was then freshly diluted to the final concentration about 30 minutes before it was added to the adipose tissue.

Established freezing and thawing protocol. A number of freezing and thawing protocols were attempted during our preliminary studies. The protocol described in this report represents the optimum for adipose tissue and was used throughout the study. For experimental group 2, 1 mL of adipose aspirates after preparation was put into a 3-mL vial and mixed with 1 mL of combined DMSO (in 0.5 M) and trehalose (in 0.2 M) solution. After adding CPAs, the vial was placed in room temperature for 10 minutes and then put into a methanol bath (Kinetics, Stone Ridge, NY). The freezing system was set up at 1˚C to 2˚C per minute of slow cooling rate from 22˚C to –30˚C without artificially-induced ice formation. The vial was then transferred to liquid nitrogen (–196˚C) after it reached –30˚C and held there for 10 minutes for long-term preservation. For experimental group 1, 1 mL of adipose aspirates after preparation was put into a 3-mL vial and mixed with 1 mL of normal saline; it was then subjected to the same freezing protocol as described above. All cryopreserved adipose aspirates, once they became equilibrated in liquid nitrogen in about 20 minutes, were considered to have undergone the equivalent of long-term preservation and were ready for thawing. Before thawing, the vial containing cryopreserved adipose aspirates was taken from the liquid nitrogen tank and placed at room temperature for 2 minutes in order to let the liquid nitrogen vapor out of the vial. The vial was then dropped into a stirred 37˚C water bath until the pre-

served adipose aspirates were thoroughly thawed. For the control group, 1 mL of adipose aspirates after centrifugation was mixed with 1 mL of normal saline and placed at room temperature for assessment.

Assessments of preservation method. Three methods were used to assess the preservation method. The first of these was a viable adipocyte cell count. One gram of adipose aspirates was taken from each of the 3 groups and washed 3 times with phosphate-buffered solution (PBS). It was then mixed with 1 mg per mL of type 1 collagenase (Sigma, St. Louis, MO) in PBS containing 0.5% bovine serum albumin (Sigma, St. Louis, MO) for digestion and was incubated at 37°C in a CO_2 incubator. After 1-hour incubation, the digestion was terminated with 10% fetal calf serum (Sigma, St. Louis, MO) and any remaining tissue fragments were removed by straining the digested fatty tissues through a piece of large-weave gauze. The digested fatty tissues were fractionated into mature adipocytes (top layer) and stromal pellet (bottom) after a centrifugation at 200 *g* for 10 minutes. The viable fatty cells were determined after 0.4% trypan blue vital stain (Sigma, St. Louis, MO) from a 100-μL sample with 1:1 dilution with trypan blue. The number of viable fatty cells was then counted with a hemocytometer under a microscope with ×400 magnification.

The second method was a glycerol-3-phosphate dehydrogenase (G3PDH) assay. G3PDH assay was chosen to assess cellular function of fatty tissues because it is relatively simple but is adipocyte-specific. According to the manufacturer's instructions (Kamiya Biomedical Co, WA), G3PDH activity within fatty tissues was evaluated using a spectrophotometric assay. Briefly, 1 gram of fresh or cryopreserved adipose aspirates was mixed with 4 mL of 0.25 M cane sugar solution and homogenized. The mixture was then spun at 700 *g* at 4°C for 10 minutes and the supernatant was taken to the special centrifuge tube, which was again spun but at 54,000 *g* for 60 minutes. The supernatant obtained after the second centrifugation was diluted approximately 20 to 100 times with an enzyme-extracting reagent. Final procedures of the assay were as follows: the substrate reagent (400 μL) was dispensed into an assay well and heated to 25°C; the diluted supernatant was also heated to 25°C and 200 μL of it added to the well and mixed with the substrate reagent; the optical absorption at 340 nm was measured for 3 to 10 minutes and plotted on a graph; the change in optical density (O OD) per minute from the linear position of the curve was obtained; and G3PDH activity was calculated based on the formula (G3PDH activity [U/mL] = O OD @ 340 nm/minute × 0.482) and the value expressed as U/mL.

Finally, the fresh or cryopreserved adipose aspirates (about 2-3 g) underwent histologic examination. The aspirates were fixed immediately in 10% buffered formalin, concentrated by gravity filtration through a porous paper ("tea bag"), processed through graded alcohols and xylene, embedded in paraffin, sectioned at 5 microns, and stained with H&E staining. All histologic slides were examined by a pathologist (MLC) in a single-blinded fashion for evidence of architectural disruption, adipocyte degeneration, or necrosis.

Statistical analysis. All data in this study were expressed as mean ± standard deviation (SD). A 2-tailed Student *t* test was used to assess the difference between the groups. A P value < 0.05 was considered statistically significant.

Results

Viable adipocyte counts were performed in all fresh or cryopreserved specimens. Although there was a low viable adipocyte count in experimental group 2 (optimal cryopreservation with CPAs) when compared with the control group (fresh specimens) (2.15 ± 0.68 vs 2.91 ± 0.89 × 10^6/mL, $P < 0.05$), the number of viable adipocytes was much higher in experimental group 2 compared with experimental group 1(simple cryopreservation) (2.15 ± 0.68 vs 1.04 ± 0.35 × 10^6/mL, $P < 0.0001$) (Figure 1).

G3PDH assay was used to assess cellular function of fatty tissues within fresh or cryopreserved adipose specimens in each group. There was no statistically significant G3PDH activity in the control group compared with the experimental group 2 (0.34 ± 0.11 vs 0.28 ± 0.09 u/mL, P = NS). However, there was statistically significant increase of G3PDH activity in the experimental group 2 compared with the experimental group 1 (0.28 ± 0.09 vs 0.17 ± 0.07 U/mL, $P < 0.01$), indicating good cellular function of fatty tissues maintained after this method of cryopreservation (Figure 2).

Routine histology with H&E staining was performed in all groups to see whether cryopreservation would alter the architecture of fatty tissues. Varying degrees of fatty tissue shrinkage were found in all cryopreserved groups (Figure 3, *A* and *B*) compared with the control group (Figure 3, *C*) but there was no evidence of fatty tissue degeneration or necrosis. The basic architecture of fragmental fatty tissues was maintained in all cryopreserved adipose specimens except for slightly more tissue shrink-

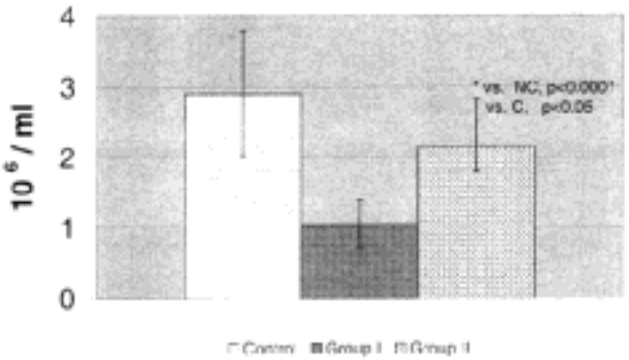

Figure 1. *Results of viable adipocyte counts. Data were expressed as mean ± SD.*

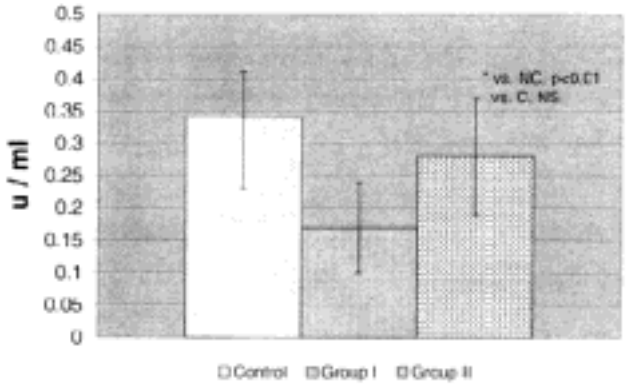

Figure 2. *Results of G3PDH assay. Data were expressed as mean ± SD.*

age found in experimental group 1 (Figure 3, *A*) compared with experimental group 2 (Figure 3, *B*).

Discussion

Adipose aspirates obtained from conventional lipoplasty could be a useful source for future autologous fat grafting, or even for tissue engineering, if long-term preservation of the aspirates becomes possible.[1,2,13] Unfortunately, an optimal technique for long-term preservation of adipose tissues is not available at the present time, and studies that investigate such a method for preservation of adipose tissue are rare and not very successful.[3,4] In this study, adipose aspirates obtained from conventional lipoplasty were preserved successfully by means of a modern cryopreservation technique with a combination of DMSO and trehalose and our established freezing and thawing protocol. Significantly more viable adipocytes and better cellular function of adipose aspirates were preserved with our optimal cryopreservation technique in comparison to a simple cryopreservation method. Although adipose aspirates preserved with our technique are not as ideal as fresh specimens, based on the number of viable adipocytes and histology, this study was the first successful report addressing the application of a modern cryopreservation technique for long-term preservation of adipose tissues.

There is no established freezing and thawing protocol for cryopreservation of adipose tissues in the literature. The protocol used in this study was based on principles from other studies in our laboratory in which organs or tissues were preserved successfully by using CPAs and controlled freezing and thawing processes.[8,14,15] Controlled slow cooling and fast rewarming were used, with the rate for both cooling and rewarming determined by our preliminary studies. Although different freezing and thawing protocols were tested at the beginning of the study, a freezing and thawing protocol with controlled slow cooling (1-2°C per minute from 22°C to –30°C) and fast rewarming to 37°C represented the best for cryopreservation of adipose tissue and was used throughout.

DMSO, one of the most commonly used CPAs in modern cryopreservation, was used in this study but at a concentration (3.3%) much lower than that used in previous reports (10%-20%).[16,17] Because DMSO can be toxic to living tissues or cells,[18] the low concentration of DMSO used in our cryopreservation medium may reduce the toxicity of CPA to adipose tissues after thawing. During our preliminary studies, we also found that by adding trehalose to the cryopreservation medium, the results for adipose aspirates after cryopreservation could be improved. The above findings were consistent with a recent report on cryopreservation of fetal skin with a combination of trehalose and DMSO as CPAs.[19]

Cryopreservation processes do not appear to alter the architecture of adipose tissues significantly. This finding may reflect the fact that adipocytes primarily filled with lipid and containing very little intracellular water undergo relatively less volume change when frozen. However, simple cryopreservation of adipose tissues without addition of CPAs demonstrated poor cellular function of adipocytes within adipose aspirates. The addition of CPAs, such as DMSO and trehalose, significantly enhanced the viability of adipose tissues after cryopreservation, with more viable adipocytes preserved and better cellular function maintained. Because intracellular ice formation is considered to be the primary mechanism by which any cell damage may occur, cryoprotectants may reduce physical injury by preventing the formation of ice crystals. A permeable CPA, like DMSO, is thought to protect cells against freezing injury by reducing ice for-

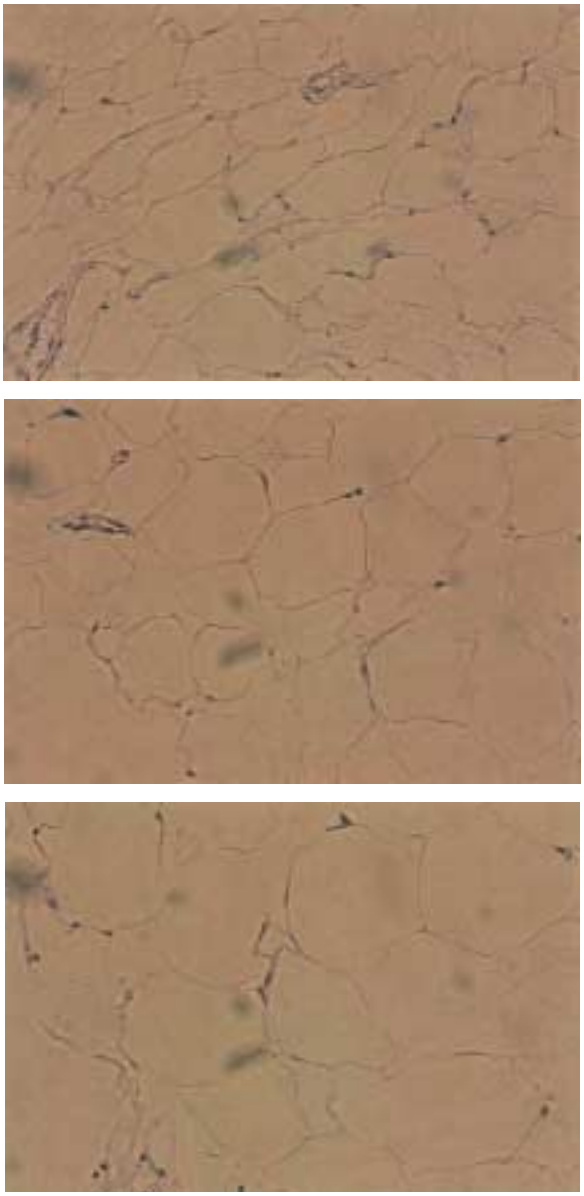

Figure 3. *Routine histology of cryopreserved adipose aspirates (H&E staining, original magnification ×200) showed that cryopreservation did not alter the architecture of fragmental fatty tissues. Only mild to moderate shrinkage of fatty tissues was observed, but their structure was basically maintained without evidence of adipocyte degeneration or necrosis.* **A,** *Experimental group 1, with simple cryopreservation.* **B,** *Experimental group 2, with optimal cryopreservation.* **C,** *Control group (fresh adipose aspirates).*

mation inside and outside the cells.[12,14] However, a nonpermeable CPA, like trehalose, may provide protection in several ways. It dehydrates cells and thus reduces the amount of water before freezing. It also stabilizes cellular membranes and proteins during freezing and drying.[19] Clearly, a combination of both types of CPAs (DMSO and trehalose) could theoretically enhance the effect of cell protection during cryopreservation. Therefore, the cryopreservation protocol developed in this study could be considered a standard technique for future study of long-term preservation of adipose tissues obtained from conventional lipoplasty.

The methods used in this study to test adipocyte viability rely on cell isolation procedures and may not accurately reflect adipose viability in the intact tissue because isolation of adipocytes has eliminated the stromal component surrounding them. Therefore, testing the viability of adipose tissues in vitro can be a difficult task and no satisfactory method has yet been established. An MTT assay has recently been introduced to test viability of free fat grafts in vitro but its reliability has not yet been confirmed by others.[20] Within our cryoprotective medium, DMSO, although in low concentration, still needs to be removed from adipose aspirates after successful cryopreservation before these tissues can be administered in vivo. DMSO within cryopreserved adipose tissues can be removed effectively after a series of subsequent dilutions with normal saline and centrifugations at room temperature.[15] However, the above techniques may not be practical and convenient enough for clinicians at the present time. Therefore, further studies are needed to develop an optimal and practical technique of cryopreservation that does not require removing CPAs from preserved adipose aspirates after thawing before in vivo administration. An in vivo study will also be conducted to confirm the results from our current in vitro study.

Conclusion

The results from this study demonstrated, for the first time, an effective method for long-term preservation of adipose aspirates by means of a modern cryopreservation approach. The combination of DMSO and trehalose as cryoprotective agents appears to provide adequate preservation of adipose tissues obtained from conventional lipoplasty. If such a method is confirmed by our further in vivo studies, long-term cryopreservation of adipose tissues can be successfully performed for possible future autologous fat grafting or can be banked as a source for human stem cells. ■

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Reprint requests: Lee L. Q. Pu, MD, PhD, Division of Plastic Surgery, University of Kentucky, Kentucky Clinic, K454, Lexington, KY 40536-0284.

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