

Cryopreservation of Adipose Tissues: The Role of Trehalose

Lee L. Q. Pu, MD, PhD; Xiangdong Cui, MD; Betsy F. Fink; 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: Several studies have shown that one of the sugars, trehalose, can improve tissue survival after cryopreservation when combined with other cryoprotective agents, and thus may possibly be used in cryopreservation of adipose tissues that have been found more resistant to injury after freezing.

Objectives: The purpose of this study was to test our hypothesis that lipoplasty-derived adipose aspirates could be effectively cryopreserved by adding trehalose as the sole cryoprotective agent (CPA), and to develop a practical technique to effectively preserve adipose tissues for future applications.

Methods: The middle layer of adipose aspirates obtained from conventional lipoplasty was collected after centrifugation and each specimen was randomized into 3 groups: the control group, fresh adipose aspirates without preservation; the simple cryopreservation group (no CPA); and the optimal cryopreservation group (with trehalose as a CPA). 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: More viable adipocytes and better cellular function of adipose aspirates were found in the optimal cryopreservation group compared to the simple cryopreservation group, but these results were less ideal than results from the control group.

Conclusions: An optimal cryopreservation method using trehalose as a CPA appears to provide better long-term preservation of adipose aspirates than a simple cryopreservation method. Further studies are needed to refine our method for cryopreservation with trehalose as a CPA and confirm our findings in vivo. (*Aesthetic Surg J* 2005;25:126-131.)

The use of autologous fat transplantation in both aesthetic and reconstructive plastic surgery has revolutionized the field of soft tissue augmentation. As lipoplasty procedures gained popularity in the past 2 decades, it was discovered that the adipose aspirates obtained from lipoplasty could provide a source of fatty tissues for autologous transplantation. These aspirates have many of the properties desirable for soft tissue augmentation: they are abundant, readily available, inexpensive, and host-compatible.^{1,2} With a growing array of plastic surgery applications for soft tissue augmentation, the option of preserving and banking already harvested fatty tissues for possible future use or repeated application is in greater demand.³

Modern cryopreservation techniques permit the long-term storage of living cells and tissues for many potential clinical applications.⁴⁻¹¹ At the present time, adipose aspirates can only be used for immediate autologous fat

grafting in the same setting as lipoplasty; therefore, adipose aspirates obtained from the procedure are usually discarded. It has been a strong desire of both plastic surgeons and patients to preserve adipose aspirates obtained from conventional lipoplasty for potential future applications.

Recently, an optimal long-term preservation method for adipose tissues was developed in our laboratory. By adding relatively low concentrations of dimethyl sulfoxide (DMSO) and trehalose as cryoprotective agents (CPAs), using a controlled slow cooling and fast rewarming protocol, good preservation of adipose tissues obtained from conventional lipoplasty has been demonstrated.¹² However, DMSO has a toxic effect on tissues and has to be removed after thawing before cryopreserved adipose tissues can be administered in vivo.¹³

Trehalose is a non-reducing disaccharide of glucose found at high concentrations in a wide variety of organ-

isms that are capable of surviving complete dehydration.¹⁴ When it is used in combination with DMSO as a CPA, the viability of cells or tissues after cryopreservation is significantly enhanced.¹⁵⁻¹⁸ This benefit is probably the result of a synergistic effect of trehalose as a non-permeable CPA to DMSO. Trehalose has also been used alone as a CPA in cryopreservation of red blood cells and sperm, with promising results.^{19,20} The purpose of the present study was to evaluate whether the use of trehalose alone as a CPA could effectively protect adipose tissues during cryopreservation. Since trehalose is basically a sugar and is not tissue toxic, it does not need to be removed from previously cryopreserved adipose tissues before they are administered in vivo.

Materials and Methods

Preparation of adipose aspirates

Adipose aspirates were obtained from specimens after conventional lipoplasty (suction-assisted lipoplasty only) performed at University of Kentucky Medical Center and Lexington Healthsouth Outpatient Surgery Center, both in Lexington, Kentucky. The study was approved by the Institutional Review Board. All specimens were obtained from the abdomens of 12 adult, white, female patients (aged 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. After centrifugation, the middle layer of adipose aspirates, 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 processed in vitro and then randomized into 3 groups: control group (fresh adipose aspirates without preservation); simple cryopreservation group (adipose aspirates preserved with our established cooling and thawing protocol but without adding CPA); and optimal cryopreservation group (adipose aspirates preserved with our protocol and with adding 0.25 M trehalose as a CPA).

Cryopreservation procedure

Choices of CPAs. In this study, trehalose, a non-permeable CPA, was selected based on previous studies.^{12,14-18} Many different concentrations of trehalose were used in the early stages of the experiment in order to find an optimal concentration for cryopreservation of adipose tissues. A concentration of 0.25 M trehalose (Sigma, St. Louis, MO) was determined to be optimal and was used later in the study. Double concentrations of 0.25 M trehalose were made first and then diluted to the final concentration about 30 minutes before it was added to the adipose tissue.

Freezing and thawing protocol. A freezing and thawing protocol developed from our previous study was used.¹² This protocol represents the optimum for adipose tissue and was used throughout the study. For the optimal cryopreservation group, 1 mL of adipose aspirate was placed into a 3-mL vial after preparation and mixed with 1 mL of trehalose (in 0.25 M) solution. After adding trehalose, 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 approximately 1 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 into liquid nitrogen (-196°C) after it reached -30°C and held there for 10 minutes for long-term preservation. For the simple cryopreservation group, 1 mL of adipose aspirate after preparation was placed into a 3-mL vial and mixed with 1 mL of normal saline; it was then subjected to the freezing protocol described above. All cryopreserved adipose aspirates, once they became equilibrated in liquid nitrogen in about 20 minutes, were considered to be equivalent to those that underwent 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 in 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 preserved adipose aspirates were thoroughly thawed. For the control group, 1 mL of adipose aspirate after centrifugation was mixed with 1 mL of normal saline and placed in room temperature for assessment.

Assessments of preservation method. The preservation method was evaluated by taking a viable adipocyte cell count, by conducting a glycerol-3-phosphate dehydrogenase (G3PDH) assay, and by histologic examina-

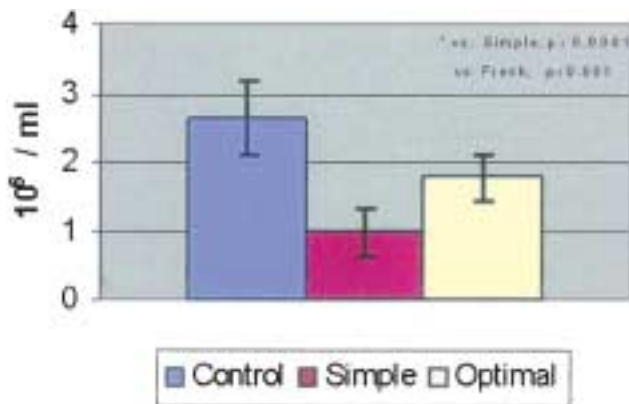


Figure 1. Results of viable adipocyte counts. Data were expressed as mean \pm SD.

tion. In the first method, 1 g of adipose aspirate was taken from all 3 groups and washed 3 times with phosphate-buffered solution (PBS). It was then mixed with 1 mg per mL of Type I collagenase (Sigma, St. Louis, MO) in PBS containing 5% bovine serum albumin (Sigma, St. Louis, MO) for digestion and incubated at 37°C in a CO₂ incubator. After 1-hour incubation, the digestion was terminated with 10% (v/v) 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- μ l sample with 1:1 dilution with trypan blue. The number of viable fatty cells was then counted with a hemocytometer under a microscope with $\times 400$ magnification.

The second method, a G3PDH assay, was chosen to assess cellular function of fatty tissues because it is relatively simple but adipocyte specific. According to the instructions from the manufacturer (Kamiya Biomedical Co., Seattle, WA), G3PDH activity within fatty tissues was evaluated using a spectrophotometric assay. Briefly, 1 g of fresh or cryopreserved adipose aspirate was mixed with 4 mL of 0.25M 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 μ l)

was dispensed into an assay well and heated to 25°C; the diluted supernatant was also heated to 25°C and 200 μ l 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 (Δ 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) = Δ OD @ 340 nm/min \times 0.482) and the value expressed as U/mL.

For the histologic examination, fresh or cryopreserved adipose aspirates (about 2 to 3 g) 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 (M.L.C.) 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 \pm standard deviation (mean \pm SD). A 2-tailed student *t*-test was used to assess the difference between the groups. A *P* value less than 0.05 was considered statistically significant.

Results

Viable adipocyte counts were performed in all fresh or cryopreserved specimens. Although the viable adipocyte count in the optimal cryopreservation group was significantly lower when compared with the control group (fresh specimens) (1.78 ± 0.33 versus $2.64 \pm 0.54 \times 10^6/\text{mL}$, $P < 0.001$), the number of viable adipocytes was much higher in the optimal cryopreservation compared with the simple cryopreservation group (1.78 ± 0.33 versus $0.99 \pm 0.35 \times 10^6/\text{mL}$, $P < 0.0001$) (Figure 1).

G3PDH assay was used in this study to assess cellular function of fatty tissues within fresh or cryopreserved adipose specimens in each group. The G3PDH activity in the control group was significantly higher when compared with the optimal cryopreservation group (0.32 ± 0.09 versus 0.24 ± 0.07 U/mL, $P < 0.05$). However, there was a statistically significant increase of G3PDH activity in the optimal cryopreservation group compared with the simple cryopreservation group (0.24 ± 0.07 versus 0.15 ± 0.06 U/mL, $P < 0.01$) indicating good cellular function of fatty tissues maintained after optimal cryopreservation (Figure 2).

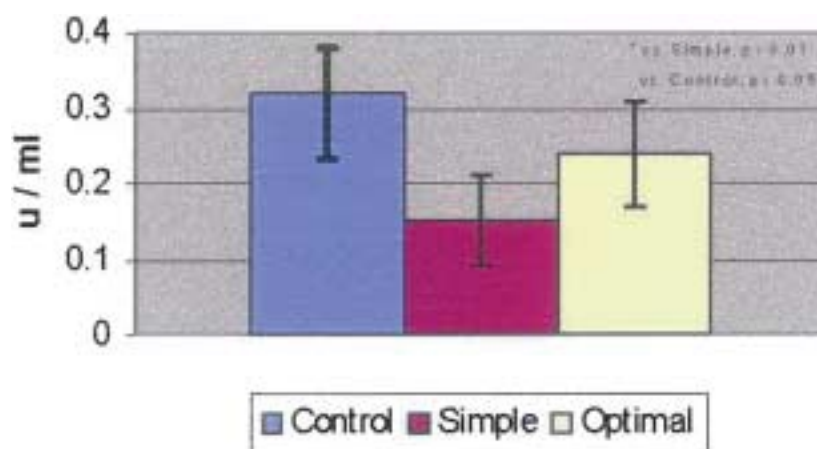


Figure 2. Results of G3PDH assay. Data were expressed as mean \pm SD.

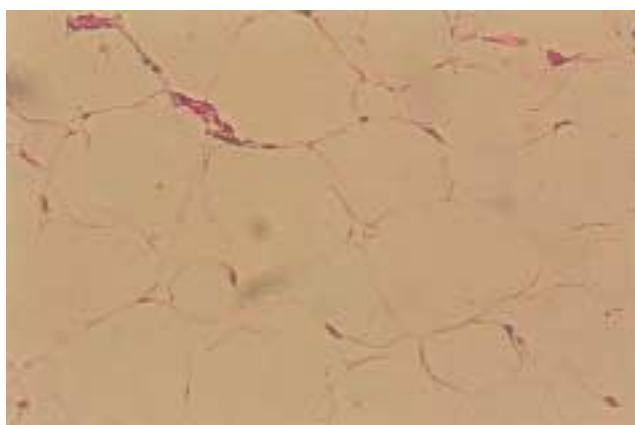


Figure 3. Routine histology of fresh adipose aspirates from the control group (H&E staining, original magnification $\times 200$) shows completely normal structure of fatty tissues.



Figure 4. Routine histology of cryopreserved adipose aspirates from the simple cryopreservation group (H&E staining, original magnification $\times 200$) shows more prominent shrinkage of fatty tissues compared to the control group. The structure of adipose tissues was maintained, with no evidence of tissue degeneration or necrosis.

Varying degrees of fatty tissue shrinkage were found in all cryopreserved groups compared with the control group (Figures 3 to 5), but there was no evidence of fatty tissue degeneration or necrosis. Cryopreservation appeared not to alter the architecture of fatty tissues significantly and the basic structure of fragmental fatty tissues was maintained in all cryopreserved adipose tissues. However, more prominent tissue shrinkage was found in the simple cryopreservation group compared with the control group (Figures 3 and 4). Such tissue shrinkage was less prominent in the optimal cryopreservation group compared with the control group (Figures 3 and 5).

Discussion

An optimal method for long-term preservation of adipose aspirates by means of a modern cryopreservation

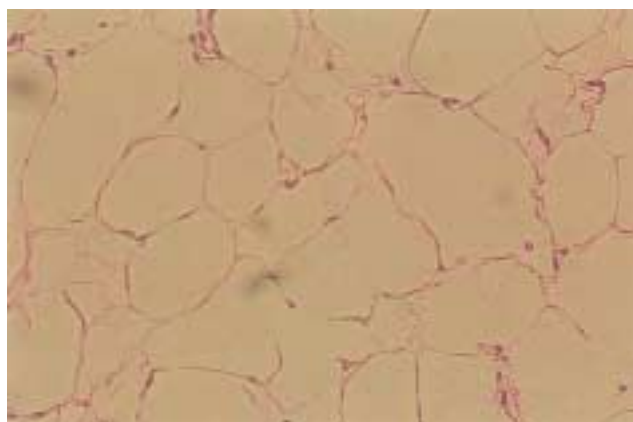


Figure 5. Routine histology of cryopreserved adipose aspirates from the optimal cryopreservation group (H&E staining, original magnification $\times 200$) shows less prominent shrinkage of fatty tissues compared to the control group. The structure of adipose tissues was maintained well, with no evidence of tissue degeneration or necrosis.

approach was recently developed in our laboratory.¹² That method involved the use of a combination of DMSO and trehalose as a CPA for cryopreservation of adipose tissues in addition to a controlled slow cooling and fast rewarming protocol. Although the addition of both CPAs appeared to provide good preservation of adipose tissues, these cryopreserved adipose tissues still could not be used clinically. One of the CPAs, DMSO, was used in low concentrations but still had to be removed from adipose tissues after successful cryopreservation before these tissues could be administered *in vivo* because of the toxic effect of DMSO on living tissues and cells.¹³ The present study was conducted specifically to evaluate whether the use of trehalose alone as a CPA would be able to provide adequate preservation of adipose tissues during cryopreservation with our established freezing and thawing protocol.

In this study, 0.25M trehalose was added to adipose tissues as a CPA. Addition of trehalose was found to improve long-term preservation of adipose aspirates collected from conventional lipoplasty. Significantly more viable adipocytes and better cellular function of adipose tissues were seen in an optimal cryopreservation group (with trehalose as a CPA) compared to a simple cryopreservation group (without a CPA). Although adipose tissues preserved with our optimal cryopreservation technique are still not as ideal as fresh adipose tissues, we believe, based on the results from this study, that trehalose could potentially be used alone, as a single CPA, in long-term preservation of adipose tissues because it does not need to be removed from previously cryopreserved tissues before they are administered *in vivo*.

Intracellular ice formation is considered to be the primary mechanism by which any cell damage may occur during or after cryopreservation. CPAs may reduce physical injury by preventing the formation of ice crystals. Although a permeable CPA like DMSO is thought to protect cells against freezing injury by reducing ice formation inside and outside the cells,^{21,22} a non-permeable CPA like trehalose may provide protection through several different mechanisms. It dehydrates cells and thus reduces the amount of water present before freezing. In addition, it stabilizes cellular membranes and proteins during freezing and drying. Because adipocytes are primarily filled with lipid and contain very little intracellular water, they may undergo relatively less volume change when frozen. The cryopreservation process, in theory, may not alter the architecture of adipose tissues significantly. Thus, application of trehalose alone as a single

CPA should provide adequate protection of adipose tissues during cryopreservation. The results from this study have confirmed this theory.

Trehalose has been used in conjunction with DMSO during cryopreservation. A combination of trehalose and DMSO greatly enhanced the effect of cell or tissue protection during cryopreservation through a possible synergistic mechanism.¹⁵⁻¹⁸ Trehalose alone was assessed as a possible substitute for glycerol (a permeable CPA) in the cryopreservation of human red blood cells (RBCs) and was found to recover an average of 81% of RBCs after a standard cryopreservation compared with glycerol, which recovered nearly 90% of RBCs. The cell morphology of RBCs was maintained with use of trehalose.¹⁹ Trehalose was also found to provide even better protection of mouse sperm after cryopreservation compared to a permeable CPA such as DMSO or glycerol, as evidenced by post-thawed sperm motility studies.²⁰ A non-permeable CPA, such as trehalose, may provide adequate protection of tissues or cells in cryopreservation if they contain less intracellular water.^{17,20}

Testing the viability of adipose tissues *in vitro* may be difficult because most available methods rely on cell isolation procedures and may not accurately reflect adipose viability in the intact tissue. An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay has recently been introduced to test viability of free fat grafts *in vitro*, but its reliability has not yet been confirmed by others.²³ The results of adipocyte counts and G3PDH assay in our optimal cryopreservation group are still less than optimal when compared to the results from the control group. Additional studies should be conducted to search for the ideal concentration of trehalose as a CPA for cryopreservation of adipose tissues. Because trehalose may be introduced into cells during cryopreservation using a genetically engineered version of α -hemolysin, a pore-forming protein,²⁴ its protective role as a CPA for tissues or cells can be enhanced during cryopreservation. Furthermore, an *in vivo* study should also be conducted to confirm the results from our present *in vitro* study.

Conclusion

The results from this study indicate that the use of trehalose as a CPA can improve long-term preservation of adipose tissues collected from conventional lipoplasty by means of a modern cryopreservation method. Several modifications will be needed to refine our technique in cryopreservation of adipose tissues. If the findings of the present study can be confirmed by our future *in vivo* studies, long-

term cryopreservation of adipose tissues can become a real option for patients who are willing to store their adipose tissues collected from conventional lipoplasty.

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References

1. Billings E Jr, May JW Jr. Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. *Plast Reconstr Surg* 1989;83:368-381.
2. Sommer B, Sattler G. Current concepts of fat graft survival: histology of aspirated adipose tissue and review of the literature. *Dermatol Surg* 2000;26:1159-1166.
3. Shoshani O, Ullmann Y, Shupak A, Ramon Y, Gilhar A, Kehat I, et al. The role of frozen storage in preserving adipose tissue obtained by suction-assisted lipectomy for repeated fat injection procedures. *Dermatol Surg* 2001;27:645-647.
4. Wagner CT, Martowics ML, Livesey SA, Connor J. Biochemical stabilization enhances red blood cell recovery and stability following cryopreservation. *Cryobiology* 2002;45:153-166.
5. Rowley SD, Feng Z, Chen L, Holmberg L, Heimfeld S, MacLeod, et al. A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethylstarch. *Bone Marrow Transplant* 2003;31:1043-1051.
6. Anger JT, Gilbert BR, Goldstein M. Cryopreservation of sperm: indications, methods and results. *J Urology* 2003;170:1079-1084.
7. Cui XD, Labarrere C, He LQ, Cheng S, Siderys H, Kovacs R, et al. Cryopreservation and microsurgical implantation of rabbit carotid arteries. *Cell Preservation Technology* 2002;1:121-128.
8. Kofron MD, Opsitnick NC, Attawia MA, Laurencin CT. Cryopreservation of tissue engineered constructs for bone. *J Orthop Res* 2003;21:1005-1010.
9. Castagnoli C, Alotto D, Cambieri I, Casimiri R, Aluffi M, Stella M, et al. Evaluation of donor skin viability: fresh and cryopreserved skin using tetrazolium salt assay. *Burns* 2003;29:759-767.
10. Shimizu K, Kumita S, Kitamura Y, Nagahama M, Kitagawa W, Akasau H, et al. Trial of autotransplantation of cryopreserved thyroid tissue for postoperative hypothyroidism in patients with Graves's disease. *J Am Coll Surg* 2002;194:14-22.
11. Nakagawa T, Yano K, Hosokawa K. Cryopreserved autologous nipple-areola complex transfer to the reconstructed breast. *Plast Reconstr Surg* 2003;111:141-147.
12. Pu LLQ, Cui X, Fink BF, Cibull ML, Gao D. Long-term preservation of adipose aspirates after conventional lipoplasty. *Aesthetic Surg J* 2004;24:541-545.
13. Pegg DE. The history and principles of cryopreservation. *Semin Reprod Med* 2002;20:5-13.
14. Crowe JH, Crowe LM, Chapman D. Prevention of membranes in anhydrobiotic organisms: the role of trehalose. *Science* 1984;223:701-703.
15. Beattie GM, Crowe JH, Lopez AD, Cirulli V, Ricordi C, Hayek A. Trehalose: a cryoprotectant that enhances recovery and preserves function of human pancreatic islets after long-term storage. *Diabetes* 1997;46:519-523.
16. Yokomise H, Inui K, Wada H, Hasegawa S, Ohno N, Hitomi S. Reliable cryopreservation of trachea for one month in a new trehalose solution. *J Thorac Cardiovasc Surg* 1995;110:382-385.
17. Erdag G, Eroglu A, Morgan JR, Toner M. Cryopreservation of fetal skin is improved by extracellular trehalose. *Cryobiology* 2002;44:218-228.
18. Choi YH, Chang YJ. The influence of cooling rate, developmental stage, and the addition of sugar on the cryopreservation of larvae of the pearl oyster *Pinctada Fucata Martensii*. *Cryobiology* 2003;46:190-193.
19. Pellerin-Mendes C, Million L, Marchand-Arvier M, Labrude P, Vigneron C. In vitro study of the protective effect of trehalose and dextran during freezing of human red blood cells in liquid nitrogen. *Cryobiology* 1997;35:173-186.
20. Sztain JM, Noble K, Farley JS, Mobraaten LE. Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. *Cryobiology* 2001;41:28-39.
21. Karlsson JOM, Toner M. Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials* 1996;17:243-256.
22. Gao DY, Critser JK. Mechanisms of cryoinjury in living cells. *Institute of Lab Animal Resources J* 2000;41:187-196.
23. MacRae JW, Tholpady SS, Katz AJ, Gampper TG, Drake DB, Ogle RC, et al. Human adipocyte viability testing: a new assay. *Aesthetic Surg J* 2003;23:265-269.
24. Chen T, Acker JP, Eroglu A, Cheley S, Bayley H, Fowler A, et al. Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells. *Cryobiology* 2001;43:168-181.

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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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