The Fate of Cryopreserved Adipose Aspirates After In Vivo Transplantation

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Background: *Successful long-term preservation of adipose tissues may have an important impact on future clinical application of autologous fat transplantation. Our group has recently developed an optimal cryopreservation method for possible long-term preservation of adipose aspirates.*

Objective: *The purpose of this study was to evaluate the fate of previously cryopreserved adipose aspirates after in vivo administration in an established nude mouse model.*

Methods: *Adipose aspirates were collected from a cosmetic lipoplasty of the patient's abdomen after centrifugation. In the fresh control group (n = 20), fresh adipose aspirates were injected into the posterior scalp of a nude mouse. In the optimal cryopreservation group (n = 20), adipose aspirates after the optimal cryopreservation were injected. In the simple cryopreservation group (n = 20), adipose aspirates after the simple cryopreservation were injected. All animals in each group were observed for gross appearance of maintained fat grafts over their posterior scalps for up to 16 weeks. The final volume and weight of maintained fat grafts and their histology were evaluated at the end of the study.*

Results: *More maintained volume, weight, and fatty tissue structure of injected free grafts were found in the optimal cryopreservation group compared with the simple cryopreservation group, but the results were still less satisfactory than those in the fresh control group.*

Conclusions: *Based on this in vivo study, we believe that an optimal cryopreservation method developed in our laboratory provides reasonably good long-term preservation of adipose aspirates. However, further studies may still be warranted to refine our method for optimal cryopreservation of adipose tissues.* (Aesthetic Surg J 2006;26:653–661.)

Autologous fat graft (AFT) is still considered to be an ideal soft tissue filler in cosmetic and reconstructive surgery because, as a graft material, it is abundant, readily available, inexpensive, and host compatible.[1,2] However, one of the main obstacles to the achievement of 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.[3,4] The high rate of absorption after AFT often necessitates either overcorrection or repeated procedures in the desired area, causing patient discomfort, less optimal appearance, dissatisfaction, cost, and morbidity or trauma of the donor site.

At the present time, adipose aspirates can only be used for immediate autologous fat grafting during the same setting of lipoplasty, and adipose aspirates obtained from the procedure are usually discarded. It has been a strong desire of both plastic surgeons and patients to be able to preserve these for potential future applications if a reliable technique could be developed for long-term preservation of adipose aspirates.

In our laboratory, living cells such as platelets[5] and living tissues such as small elastic arteries[6] have been preserved with an established cryopreservation method. More recently, adipose aspirates obtained from a conventional lipoplasty have also been preserved successfully in vitro with an optimal cryopreservation method developed specifically for adipose tissues.[7] To the best of our knowledge, there are no successful in vivo studies on the long-term preservation of autologous fatty tissues for future repeated transplantations. Our technique has now made it possible to potentially bank adipose aspirates for future applications once they are harvested. The present study was therefore conducted to evaluate the adminis-

tration of previously cryopreserved adipose aspirates in a nude mouse model after in vivo transplantation in preparation for possible future repeated autologous fat grafting in a clinical setting.

Materials and Methods

Collection and processing of adipose aspirates

Adipose aspirates were obtained from a healthy 43-year-old white woman who underwent conventional suction-assisted lipoplasty of her abdomen for cosmetic contour improvement at the University of Kentucky Chandler Medical Center, Lexington, KY. The study was approved by the University's institutional review board. The abdomen of the patient was infiltrated with 2 L of lactated Ringer's solution with 30 mL of 2% lidocaine and 1 mL of epinephrine prior to the procedure. Byron aspiration cannulas (3 or 4 mm in diameter) and a conventional lipoplasty machine (Byron Medical, Tucson, AZ) were used to harvest adipose aspirates in this study. Only 100 mL of adipose aspirates were collected in a sterile bottle at the time of the lipoplasty, and the bottle was transferred immediately to the laboratory for centrifugation at 50 g for 10 minutes on a large-capacity centrifuge (Mistral 3000i, Dearborn, MI). The bottom layer of adipose segment, which usually contains more viable adipocytes,[8] was then pipetted from the large test tube. From this layer, each 20 mL of adipose aspirates was used to conduct the following experiment according to our research protocol.

Experimental design

Adipose aspirates from the patient after centrifugation were randomized into the following groups: (1) fresh adipose aspirates without preservation (the fresh control group); (2) adipose aspirates preserved with cryoprotective agents (CPAs) (the optimal cryopreservation group); and (3) adipose aspirates preserved with liquid nitrogen only without CPAs (the simple cryopreservation [negative control] group).

Cryopreservation technique

Choices of CPAs. In this study, dimethyl sulfoxide (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.[9,10] The optimal combination, consisting of 0.5 M (3.3%) DMSO (Sigma, St. Louis, MO) and 0.2 M trehalose (Sigma, St. Louis, MO), was determined from our previous in vitro study[7] and was used 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 adipose tissues.

Our established freezing and thawing protocol. The protocol described in this report was based on our previous in vitro study7 and was used throughout the study. For the optimal cryopreservation group, 1 mL of adipose aspirates after preparation was placed 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 was held there for 10 minutes for long-term preservation. For the simple cryopreservation group, 1 mL of adipose aspirates after preparation was placed into a 3-mL vial and mixed with 1 mL of normal saline; it was then subjected to the same freezing protocol as previously described. All previously cryopreserved adipose aspirates, once they became equilibrated in liquid nitrogen in about 20 minutes, were considered to be equivalent to ones after 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 to let the liquid nitrogen vapor out of the vial. The vial was then dropped into a stirred 37°C water bath until the cryopreserved adipose aspirates were thoroughly thawed. The DMSO within the cryopreserved adipose aspirates of the optimal cryopreservation group was removed with a series of subsequent dilutions and centrifugations.[5]

For the fresh control group, 1 mL of adipose aspirates after centrifugation was mixed with 1 mL of normal saline and placed at room temperature. All fresh or cryopreserved adipose aspirates were then ready to be injected into nude mice.

In vivo administration of cryopreserved adipose aspirates

In this study, an athymic nude mouse was selected because of its limited ability to reject foreign antigenic grafts. This animal model allows evaluation of the injected human fat grafts in a nude mouse, thus imitating the clinical situation in the best possible way.[11] Each animal was anesthetized by inhalation of isofluorane during the procedure. The back of the scalp was cleaned with an

alcohol swab. About 1 mL of fat suspension was injected into the posterior scalp via a 1-mL syringe connected with a 16-gauge needle according to our experimental protocol (fresh adipose aspirates for the fresh control group, adipose aspirates after cryopreservation with CPAs for the optimal cryopreservation group, and adipose aspirates after cryopreservation without CPAs for the simple cryopreservation group). The exact preinjection volume of the adipose aspirates to be injected was recorded, and the exact weight was also measured with a balance (Mettler PM100, Mettler Inc, Hightown, NJ). A total of 20 nude mice were injected in each group, and each animal was observed for up to 16 weeks.

Assessments of fresh or cryopreserved adipose aspirates

At 16 weeks after in vivo transplantation of adipose aspirates, each animal that survived the study was euthanized. An encapsulated fat graft over the back of the mouse's scalp was removed surgically for the following assessments.

Volume and weight measurement. An encapsulated fat graft harvested from each animal was measured for volume (in cubic centimeters) by a volume displacement test. Briefly, the graft was placed into an empty 1-cc syringe, and the syringe was then filled with normal saline up to 1 mL. Once the level of the solution became stable in an upright position, the graft was gently removed with a small forceps. The actual volume of the graft (in cubic centimeters) was calculated by 1 mL minus the reading (in cubic centimenters) over the syringe without the graft.

An encapsulated "fat graft" harvested from each animal was also weighed with a balance (Mettler PM100, Mettler Inc, Hightown, NJ). The weight of each fat graft (g) was recorded.

Histologic examination. Each encapsulated fat graft harvested was fixed immediately in 10% buffered formalin. It was then processed through graded alcohols and xylene, embedded in paraffin, sectioned at 5 Ìm, and stained with H&E staining. All histologic slides were examined by an experienced pathologist (MLC) in a single-blinded fashion for evidence of architectural disruption, adipocyte degeneration, fibrosis, or necrosis.

Statistical analysis

All data in this study were expressed as mean ± SD. A two-tailed Student *t* test was used to assess the difference between the groups (Microsoft Excel 2003, Seattle, WA). A P value < .05 was considered statistically significant.

Results

In this study, 2 animals from the fresh control group, 2 from the simple cryopreservation group, and 1 from the optimal cryopreservation group died before completion of the study, and thus were excluded from the study. The fat grafts were successfully maintained during a 16-week observation after injection of fresh or cryopreserved adipose aspirates into a nude mouse's posterior scalp in the fresh control group (n = 18), the optimal cryopreservation group (n = 18), and the simple cryopreservation group (n = 19), as observed clinically by well-defined subcutaneous lumps on the animal's posterior scalp. At 8 weeks, there appeared to be more maintained fat grafts in the fresh control group, slightly less maintained red fat grafts in the optimal cryopreservation group, but obviously less maintained fat grafts in the simple cryopreservation group by gross inspection (Figure 1). At 16 weeks, the differences among the 3 groups remained similar.

The volume of injected fat grafts was 1.02 ± 0.04 mL in the fresh control group, 1.00 ± 0.04 mL in the optimal cryopreservation group, and 1.00 ± 0.04 mL in the simple cryopreservation group. The retained volume at 16 weeks when the fat grafts were harvested was 0.51 ± 0.25 mL in the fresh control group, 0.35 ± 0.08 mL in the optimal cryopreservation group, and 0.07 ± 0.04 mL in the simple cryopreservation group. When these data were expressed as the percentage of the injected fat grafts maintained (retained volume divided by preinjection volume × 100%), there was significantly higher maintained volume in the fresh control group (47.7% ± 18.6%) compared to either the optimal cryopreservation group (35.3% ± 7.8%, P < .05) or the simple cryopreservation group. (6.5% ± 3.7%, P < .0001) However, there was a significantly higher maintained volume in the optimal cryopreservation group compared to the simple cryopreservation group (P < .0001) (Figure 2).

The weight of injected fat grafts was 1.00 ± 0.04 g in the fresh control group, 0.98 ± 0.06 g in the optimal cryopreservation group, and 0.97 ± 0.05 g in the simple cryopreservation group. The retained weight at 16 weeks when the fat grafts were harvested was 0.47 ± 0.20 g in the fresh control group, 0.31 ± 0.08 g in the optimal cryopreservation group, and 0.05 ± 0.03 g in the simple cryopreservation group. When these data were expressed as the percentage of the injected fat grafts maintained (retained weight divided by preinjection weight × 100%), there was a significantly higher maintained weight in the fresh control group (45.2% ± 14.7%) compared to either the optimal cryopreservation group

Figure 1. *Gross appearance of the maintained volume over the posterior scalp of a nude mouse at 8 weeks after in vivo administration of fresh or cryopreserved adipose aspirates.* **A,** *Well-maintained fat grafts lump in the fresh control group.* **B,** *Reasonably well-maintained fat grafts lump in the optimal cryopreservation group.* **C,** *Poorly-maintained fat grafts lump in the simple cryopreservation group.*

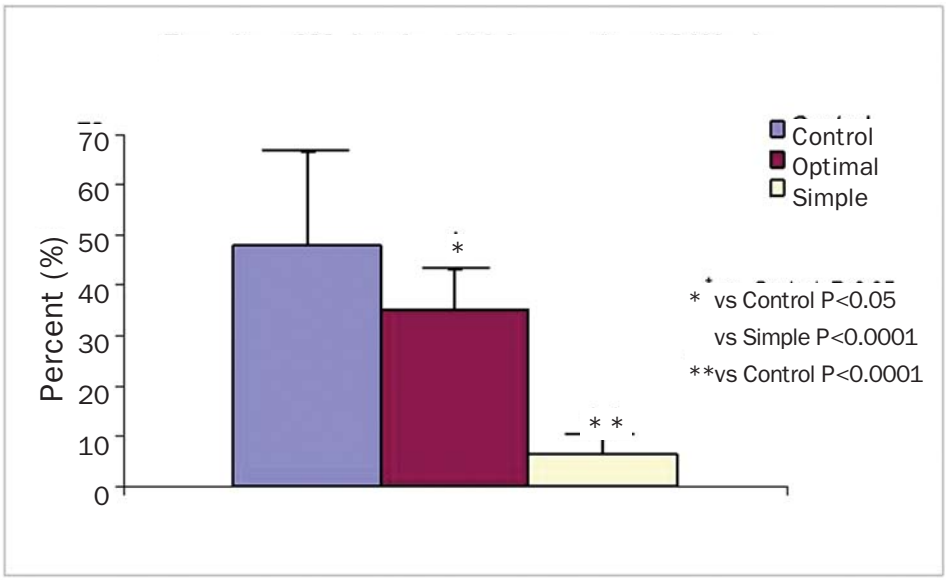

Figure 2. *Results of maintained volume after 16 weeks, expressed as percentage of maintained volume (retained volume divided by preinjection volume × 100%), at 16 weeks after in vivo administration of fresh or cryopreserved adipose aspirates.*

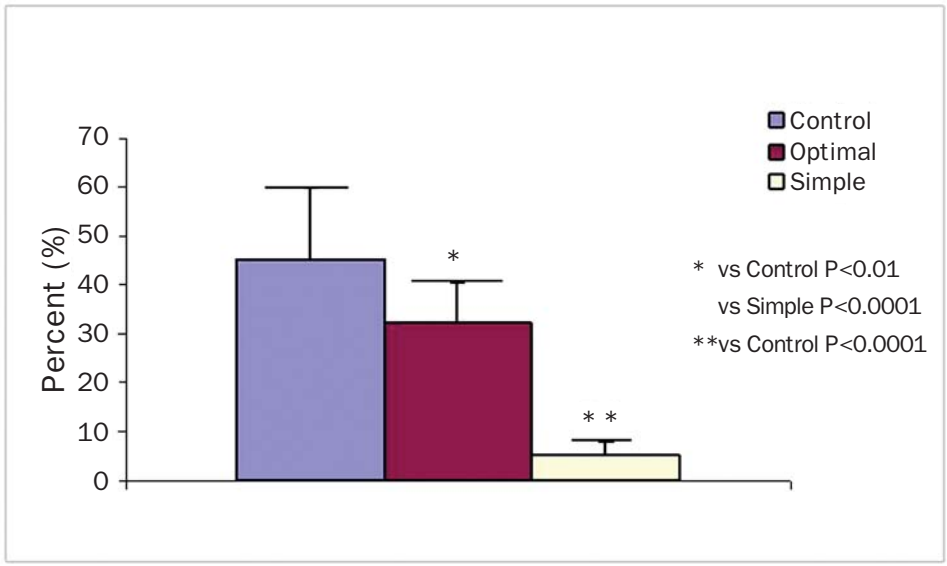

Figure 3. *Results of maintained weight after 16 weeks, expressed as percentage of maintained weight (retained weight divided by preinjection weight × 100%), at 16 weeks after in vivo administration of fresh or cryopreserved adipose aspirates.*

(32.0% ± 8.4%, $P < .01$) or the simple cryopreservation group (5.0% ± 2.7%, $P < .0001$). However, there was a significantly higher maintained weight in the optimal cryopreservation group compared to the simple cryopreservation group ($P < .0001$, Figure 3).

Histologically, a maintained fatty tissue structure with a well-organized fibrous capsule was seen in both the fresh control and the optimal cryopreservation groups. Only a relatively small amount of tissue fibrosis and fatty tissue degeneration were evidenced in the optimal cryopreservation group. However, a significantly large amount of tissue fibrosis and fatty tissue degeneration and a well-organized fibrous capsule were seen in the simple cryopreservation group (Figure 4, *A*-C).

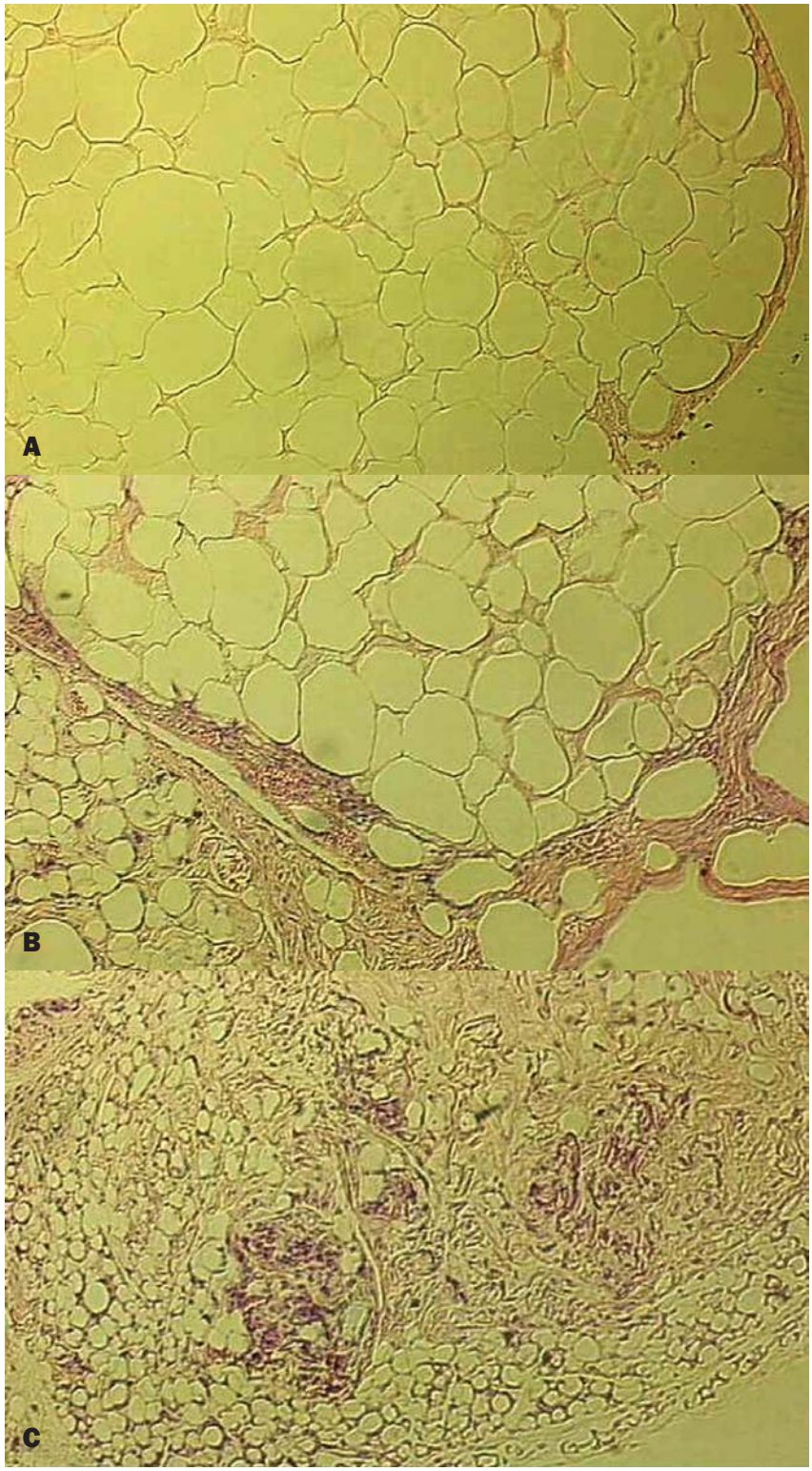

Figure 4. *Routine histology of the fat grafts at 16 weeks after in vivo administration of fresh or cryopreserved adipose aspirates (H&E staining, original magnification × 400). A well-organized fibrous capsule was seen in all 3 groups.* **A,** *Well-maintained fatty tissue structure was evidenced in the fresh control group.* **B,** *Reasonably well-maintained fatty tissue structure was also evidenced in the optimal cryopreservation group. Only a relatively small amount of tissue fibrosis and degeneration was seen.* **C,** *A significantly large amount of tissue fibrosis and degeneration was found in the simple cryopreservation group.*

Discussion

The present in vivo study was conducted based on the findings from our previous in vitro study.[7] In that study, a protocol for optimal cryopreservation of adipose tissues was successfully developed and was therefore used in this study. Our protocol emphasizes a slow cooling (1°C to 2°C per minute from room temperature to –30°C) and fast rewarming (from almost –196°C to 37°C within a few minutes). The combined use of DMSO (in 0.5 M) and trehalose (in 0.2 M) as CPAs is also a critical step in our protocol and has contributed significantly to the optimal cryopreservation of adipose aspirates collected from conventional lipoplasty.

Our in vivo study clearly demonstrated the beneficial effect of a combined use of DMSO and trehalose when comparing the results of gross inspection, maintained volume and weight, and histology between the optimal cryopreservation group and the simple cryopreservation group. Although the results from the optimal cryopreservation group were still less satisfactory than those of the fresh control group, our preferred protocol may become a possible method used clinically for optimal long-term preservation of adipose tissues harvested with the aspiration technique. To the best of our knowledge, this is the first report in the literature on successful long-term preservation of adipose tissues evaluated in an established in vivo animal model.

The results from this study again demonstrated the importance and value of adding CPAs for optimal cryopreservation of adipose tissues. This concept has not yet been formally applied to adipose tissues for long-term preservation. Although some investigators had proposed to store adipose tissues simply at –196°C in liquid nitrogen only for long-term preservation,[12] our findings clearly revealed unfavorable results of such an approach when comparing the simple cryopreservation group with the optimal cryopreservation group. A recent report by Wolter et al[13] concluded that adipocyte survival could be improved by addition of CPA. Their study again demonstrated the importance and value of adding CPAs in cryopreservation of adipose tissues and further confirmed the findings from our previous in vitro study.[7]

An athymic nude mouse was used in this study because of its limited ability to reject foreign antigenic grafts. This animal model would allow evaluation of the fate of human fat grafts after administration in vivo, and has been used to evaluate the survival of aspirated human adipose tissues injected in vivo.[11,14] Since normally there is no subcutaneous tissue in the posterior scalp, this animal model can be used to quantitatively examine the fate of human fat grafts after injection. Furthermore, about 50% of the volume loss of the injected adipose aspirates at 16 weeks in this model may be more analogous to a clinical situation in which a similar rate of the volume loss would possibly be seen within 1 year after autologous fat injection.[3,11] However, a well-organized fibrous capsule around injected fat grafts seen in all 3 groups of this study, and in studies by others using the same animal model, should not be seen in humans after autologous fat transplantation because these survived fat grafts, in theory, should have been integrated into host tissues.[1-3,11,14]

Dimethyl sulfoxide has been widely used as an effective CPA in cryopreservation of living cells or tissues.[9,10,15] The concentration of DMSO, when used alone as an CPA, is usually 10%. Because this agent is tissue toxic at normal temperature, it should be completely removed from the previously cryopreserved cells or tissues after thawing. In this study, we attempted to lower the concentration of DMSO used in cryopreservation of adipose aspirates by adding another non-tissue toxic CPA, trehalose. Although the concentration of DMSO used in this study was relatively low (0.5M, 3.3%), DMSO within previously cryopreserved adipose aspirates was removed through a series of subsequent dilutions and centrifugations because of its potential tissue toxicity at room temperature.[15]

Obviously, it should be easier to remove a low concentration of DMSO than a higher concentration from previously cryopreserved tissues and would cause less tissue damage during the process of removal from the cryopreserved tissues. A combination of trehalose, a nonpermeable CPA, with DMSO, a permeable CPA, may significantly enhance the protective effect of adipose tissues during optimal cryopreservation through a possible synergistic mechanism.[16,17] Therefore, the concentration of DMSO can be theoretically reduced when it is used in combination with trehalose. The combined use of these 2 CPAs may be valuable for optimal cryopreservation of adipose tissue or other type of tissues.

The fat grafts used in this study were harvested by conventional lipoplasty, and these adipose aspirates would normally be discarded after the procedure. This method is certainly not applicable to clinical practice where a much less traumatic technique, such as the Coleman technique, should be used.[1,2] However, based on the previous reports, significant viable adipocytes are found with adipose aspirates harvested by conventional lipoplasty after a low-force centrifugation.[8,18] Therefore, fat grafts harvested by conventional lipoplasty were used

in this study as an alternative to fat grafts harvested by the Coleman technique.

Based on the results from our previous in vitro study,[7] the DMSO and trehalose were chosen as CPAs in this study. A permeable CPA such as DMSO is thought to protect cells against freezing injury by reducing ice formation inside and outside the cells.[10,15] However, a nonpermeable CPA such as trehalose may provide protection in several ways. It dehydrates cells and thus reduces the amount of water prior to freezing. It also stabilizes cellular membranes and proteins during freezing and drying.[17] The combination of both types of CPAs (DMSO and trehalose) could theoretically enhance the effect of cell protection during cryopreservation. Therefore, this cryopreservation protocol can be considered as a standard method for future clinical application of long-term preservation of autologous fat grafts.

In this study, the results from the optimal cryopreservation group were still less satisfactory than those from the fresh control group. This may still be true not only for adipose tissue but also for other types of tissues after an optimal cryopreservation.[7,19-21] Several refinements may be needed to improve the viability of adipose tissue during a cryopreservation. For example, a specific CPA or a different combination of available CPAs may be discovered for maximal protection of adipose tissue during cryopreservation. Clearly, further studies would still be warranted to develop a reliable and clinically feasible cryopreservation method that can be used for successful long-term preservation of adipose tissue.

Conclusion

The results from our in vivo study demonstrated better maintained volume, weight, and histologic structure of adipose aspirates in the optimal cryopreservation group, where DSMO and trehalose were both used as CPAs, compared to the simple cryopreservation group. Although the results from the optimal cryopreservation group were still less satisfactory than the those from the fresh control group, long-term preservation of adipose aspirates with our established protocol may become a real option for patients who desire to bank their adipose aspirates for possible future autologous fat transplantation. ■

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