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The Search for a Useful Method for the Optimal Cryopreservation of Adipose Aspirates: Part I. In Vitro Study

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BACKGROUND: Previous studies have shown that trehalose, when used alone as a cryoprotective agent (CPA), can maintain the viability of living tissue after cryopreservation and therefore may be used clinically for the future banking of human fat grafts.

OBJECTIVE: The purpose of this study is to determine the optimal concentration of trehalose used as a CPA for the cryopreservation of adipose aspirates in vitro.

METHODS: Adipose tissue was harvested with conventional liposuction from the abdomens of eight female patients and adipose aspirates were then collected from the resulting middle layer after centrifugation. A 3-cc specimen of adipose aspirates was cryopreserved using trehalose as a CPA in seven different concentrations (0.20, 0.25, 0.30, 0.35, 0.40, 0.50, and 0.75 mol/L, respectively). Cryopreservation was conducted with controlled slow cooling to –196°C and fast re-warming to 37°C, according to our established protocol. A 3-cc specimen of fresh adipose aspirates without cryopreservation served as the control. Fresh or cryopreserved adipose aspirates in each group were evaluated by viable adipocyte counts, glycerol-3-phosphate dehydrogenase (G3PDH) assay, and histology.

RESULTS: More viable adipocytes of cryopreserved adipose aspirates were found when a 0.35 mol/L concentration of trehalose was used, as compared to the other cryopreserved groups. In terms of viable adipocyte counts, there was no statistical difference between the fresh control group and the cryopreserved group with trehalose in that concentration (1.88 ± 0.61 vs. 2.4 ± 0.52 × 10^6/mL; P > .05). G3PDH assay was performed to assess intracellular function and showed no statistical significance in the fresh control group compared with all cryopreserved groups (all P > .05). Histologically, the basic structure of adipose tissue was adequately maintained in most of the cryopreserved groups.

CONCLUSIONS: Trehalose as a CPA with a concentration of 0.35 mol/L appears to provide the optimal protection of adipose aspirates during cryopreservation. Further in vivo study will be needed to confirm these findings. (Aesthetic Surg J 2009;29:248–252)

Autologous fat can serve as the ideal filler in cosmetic or reconstructive surgery for soft tissue augmentation. However, the high rate of unpredictability of absorption following fat grafting (≤70%) usually results in repeated and frequent procedures, causing the patient to have discomfort, a less than optimal appearance, morbidity or trauma of the donor site, and potentially increased the financial costs. It is obviously desirable to both surgeons and patients if fat grafts harvested from the initial surgery can be safely banked for future applications.

At the present time, no available technique can be used clinically to bank adipose tissue for potential future applications. Our research group has studied a number of techniques for the cryopreservation of different cells and tissues, including human adipose tissue. We have found that the addition of a cryoprotective agent (CPA) to adipose tissue before cooling is a critical step in the optimal cryopreservation of human fat grafts. The most important and widely used permeable CPA in cryopreservation of living tissues is dimethyl sulfoxide (DMSO). It can protect the tissue from freezing injury and can help to achieve maximal survival of the tissue during the freezing and thawing process. However, DMSO should usually be removed from the tissue after thawing before in vivo administration because it is toxic to the tissue at room temperature.

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More recently, a freezing and thawing protocol for the cryopreservation of adipose tissue has been developed in our laboratory. With the addition of a relatively low concentration of DMSO and trehalose as CPA, good long-term preservation of adipose tissue has been shown. Unfortunately, DMSO should still be removed after thawing and before the cryopreserved fat grafts can be administered in vivo, but a process to remove DMSO within previously cryopreserved adipose tissue itself is not clinically feasible at the present time.

Trehalose is a nonreducing disaccharide of glucose and is found at high concentrations in a wide variety of organisms that are capable of surviving complete dehydration. Recent studies have shown acceptable outcomes with the use of only trehalose as a CPA in the cryopreservation of different tissues. If satisfactory preservation was achieved using trehalose alone as a CPA at an optimal concentration, human fat grafts could be banked and used later without the need for removal of the CPA from the cryopreserved fat grafts after thawing, before in vivo administration. Therefore, the purpose of the present study is to determine an optimal concentration of trehalose that can be used effectively as a CPA in the cryopreservation of adipose tissue in vitro.

MATERIALS AND METHODS

Harvesting and Preparation of Adipose Aspirates

Adipose tissue was harvested with conventional liposuction from the abdomens of eight healthy adult female patients who had no major systemic metabolic or lipid disorders. The adipose tissue was collected in a sterile bottle and transferred to our laboratory within one hour after liposuction. The specimens were then spun at 5000 g for 10 minutes on a large capacity centrifuge (MISTRAL 3000i; Curtin Matheson Scientific, Houston, TX) to separate the adipose aspirates from the 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 (22°C) unless otherwise noted and the study was approved by the University’s Institutional Review Board.

Preparation of Cryoprotective Agent Solutions

Trehalose (Sigma, St. Louis, MO) was used as a CPA for this series of experiments. The trehalose solution in different concentrations was made, filtered (0.22 μm), and stored at 4°C prior to harvesting. In general, the double-concentrated solutions were made as a stock solution and were used in a 1:1 volume mixture of CPA and adipose tissue to reach the final concentrations (0.2, 0.25, 0.3, 0.35, 0.4, 0.5, and 0.75 mol/L, respectively) before cryopreservation.

Cryopreservation Procedure

The freezing and thawing protocol was previously described. In brief, 1 mL of adipose aspirates from each patient was mixed with 1 mL of trehalose solution (in seven different concentrations; n = 6 for each concentration) in a 3-cc cryovial that was placed at room temperature for 10 minutes. Each vial was then put into a methanol bath (Kinetics, Stone Ridge, NY) for a programmed freezing. The freezing system was set for a slow cooling rate of 1°C to 2°C per minute, from 22°C to –30°C without artificially induced ice formation. When the temperature within the bath reached –30°C, it was maintained for an additional 10 minutes. Then each cryovial was transferred to liquid nitrogen at –196°C for long-term preservation. Once they became equilibrated in liquid nitrogen for 20 minutes, all cryopreserved adipose aspirates were considered to have undergone the equivalent of long-term preservation and were ready for thawing.

For thawing, each cryovial containing the cryopreserved adipose aspirates was taken from the liquid nitrogen tank and placed at room temperature for two minutes in order to allow the liquid nitrogen to vapor out of the vial. The vial was then directly dropped into a stirred 37°C water bath until the frozen adipose aspirates were thoroughly thawed. For the fresh control group, 1 mL of adipose aspirates from the same patient in each group was mixed with 1 mL of normal saline instead of a trehalose solution (n = 6) in a 3-cc cryovial and placed at room temperature for assessment.

Assessments of Cryopreservation

Viable adipocyte count. One gram of adipose aspirates was mixed with type I collagenase (Sigma; 1 mg/mL) in phosphate-buffered saline containing 5% bovine serum albumin (BSA; Sigma) for digestion and was incubated at 37°C in a CO₂ incubator. After one hour of incubation, the digestion was terminated with 1.0 mL of 10% (vol/vol) fetal calf serum (Sigma) and 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 layer) after centrifugation at 200 g for 10 minutes. The viable fatty cells were determined after 0.4% Trypan blue (Sigma) vital staining and were counted under an optical microscope.

Glycerol-3-phosphate dehydrogenase assay. Glycerol-3-phosphate dehydrogenase (G3PDH) assay was chosen to assess the cellular function of fatty tissues because it is relatively simple, but also adipocyte-specific. According to the manufacturer’s instructions (Kamiya Biomedical, Seattle, WA), G3PDH activity within adipose aspirates was evaluated using a spectrophotometric assay. In brief, 1 g of fresh or cryopreserved adipose aspirates was mixed with 4 mL of 0.25 mol/L cane sugar solution and homogenized. The mixture was then spun at 700 g for 10 minutes at 4°C and the supernatant was taken to the special centrifuge tube, which was again spun, but at 54000 g for 60 minutes at 4°C. The supernatant obtained after the second centrifugation was diluted approximately 20 to 100 times with an enzyme-
Viable adipocytes in different trehalose groups. Data are expressed as mean ± standard deviation. The highest viable adipocyte count was found in the trehalose group with a concentration of 0.35 mol/L. *Versus the trehalose group at 0.35 mol/L (P < .05); versus all other trehalose cryopreserved groups (P < .001).

Glycerol-3-phosphate dehydrogenase assay activities in different trehalose groups. Data are expressed as mean ± standard deviation. *Versus all trehalose cryopreserved groups (P > .05).

Viable Adipocytes in Different Trehalose Groups

G3PDH Activities in Different Trehalose Groups

Figure 1. Viable adipocytes in different trehalose groups. Data are expressed as mean ± standard deviation. The highest viable adipocyte count was found in the trehalose group with a concentration of 0.35 mol/L. *Versus the trehalose group at 0.35 mol/L (P > .05); versus all other trehalose cryopreserved groups (P < .001).

Figure 2. Glycerol-3-phosphate dehydrogenase assay activities in different trehalose groups. Data are expressed as mean ± standard deviation. *Versus all trehalose cryopreserved groups (P > .05).

Statistical Analysis

All data in this study were expressed as mean ± standard deviation (SD). A repeated analysis using the mixed procedure in SAS software (SAS, Cary, NC) was performed to determine whether there was a significant difference between at least two of the study groups. To further investigate this significance, a post hoc pair-wise comparison was performed using a Bonferroni correction to control for type I error. Upon finding that there was a significant difference between at least two of the groups, a post hoc pair-wise comparison was performed using a Bonferroni correction for multiple comparisons. Probabilities of a significant difference were determined from the final processed results. Unless otherwise indicated, P < .05 was considered statistically significant.

RESULTS

Viable Adipocyte Counts

Viable adipocyte counts were performed in the fresh control group and all cryopreserved groups. Among all seven cryopreserved groups, the higher viable adipocyte count was found when trehalose was at a concentration of 0.35 mol/L. There was no statistical difference in the number of viable adipocytes in this cryopreserved group compared with the fresh control group (1.88 ± 0.61 vs. 2.4 ± 0.52 × 10⁶/mL; P > .05). However, the number of viable adipocytes was statistically significantly lower in the rest of the cryopreserved groups compared with the fresh control group (all P < .001; Figure 1).

Glycerol-3-Phosphate Dehydrogenase (G3PDH) Assay

G3PDH assay was also performed in the fresh control group and all cryopreserved groups. This assay was used to assess the intracellular function of fat tissues within fresh or cryopreserved adipose aspirates. Higher levels of enzyme activity indicate better cellular function of adipocytes within the adipose aspirates. Results showed that there was no statistical difference of G3PDH activity in the fresh control group (0.35 ± 0.08 U/mL) compared with all other cryopreserved groups (all P > .05). However, among all seven cryopreserved groups, it appeared that G3PDH activity was slightly higher when the concentration of trehalose was ≥ 0.35 mol/L, but this trend did not reach statistical significance (all P > .05; Figure 2).

Histologic Examination

Routine histology with hematoxylin–eosin stain was performed in the fresh control group and all cryopreserved groups, to see whether cryopreservation would alter the architecture of fatty tissue. There was no evidence of fatty tissue degeneration or necrosis in the fresh control group or in the cryopreserved groups with different concentrations of trehalose. The basic architecture of fragmental fatty tissues was seen in the fresh control group (Figure 3) and all cryopreserved groups, except for slight tissue shrinkage found in some cryopreserved groups. The basic structure of cryopreserved adipose aspirates appeared to be adequately maintained in most of the cryopreserved groups (Figure 3).

DISCUSSION

For the last two decades, the cryopreservation of variable living cells and tissue has been studied extensively for possible clinical applications. At the present time, the cryopreservation of human reproductive cells, embryos, and
blood cells has become common practice in clinical medicine.\textsuperscript{12-14} However, very few recent studies have discussed the cryopreservation of human adipose tissue and there is no established technique for the successful cryopreservation of human fat grafts. Several recent studies in this particular area of research have been conducted by our research group, with promising results.\textsuperscript{4-6}

DMSO is a widely-used, permeable CPA for the cryopreservation of living cells and tissues. Although it provides effective protection from freezing injuries under low temperatures, it can be toxic to living cells and tissues when being held at room temperature for a prolonged period of time after thawing. DMSO can also be kept within the cells or tissues for a long period of time.\textsuperscript{7,15} In previous studies, we tried a lower concentration (0.5 mol/L) of DMSO as a CPA and we also added trehalose as a second CPA for the cryopreservation of human adipose aspirates, both with promising results.\textsuperscript{4-6} These findings were confirmed by others using similar chemical combinations.\textsuperscript{16} However, through a series of dilutions, DMSO must still be removed from the previously cryopreserved adipose aspirates after thawing to reduce or avoid its toxicity to the tissue, if the cryopreserved adipose aspirates are to be administered in vivo.\textsuperscript{3-6}

Trehalose, a natural disaccharide sugar found in many organisms, has become an attractive CPA in recent years because of its extraordinary capability to preserve the structural integrity of freezing cells.\textsuperscript{17,18} In the present study, we used only trehalose only as a CPA for the cryopreservation of human adipose tissue. A total of seven different concentrations ranging from 0.2 to 0.75 mol/L of trehalose were tested for the best possible preservation of tissue adipocyte counts and cellular function. The findings from the present study showed that trehalose at a concentration of 0.35 mol/L had the highest viable adipocyte count among all seven concentrations. The number of viable adipocytes in this group was not significantly different from the fresh control group ($P > .05$).

The number of viable adipocytes in all other cryopreserved groups was significantly lower than the fresh control group ($P < .001$). The status of cellular function, determined by intracellular activity of G3PDH, appeared to be superior with an increased concentration of trehalose, although it did not reach statistical significance because of the small sample size.

Although the true mechanism of the findings in this study remains unknown, it can be postulated that the protective effect of trehalose as a CPA for cryopreserved adipose tissue may become more efficient when it reaches an optimal concentration, such as 0.35 mol/L. Higher concentrations of trehalose (for example, those > 0.35 mol/L) may not be necessary to provide better cryopreservation of adipose aspirates. The histologic study confirmed our previous findings that trehalose caused no obvious changes in the architecture of the adipose tissue, although slight shrinkage of the adipocytes could be found in some specimens of cryopreserved adipose tissue.\textsuperscript{3-6}

Adipose tissue has some special features because adipocytes are mainly filled with lipids, have very few organelles, and have almost no water inside. A key issue for the cryopreservation of adipose tissue is preserving the integrity of the cell membrane while preventing intracellular ice formation. As shown in previous studies,\textsuperscript{19-21} trehalose can be used effectively as a CPA in the cryopreservation of cells or tissues. In the present study, with trehalose as a CPA in its concentration of 0.35 mol/L, our findings are encouraging and comparable to those demonstrated in our previous studies with a combination of DMSO and trehalose.\textsuperscript{4,6} Unlike DMSO, trehalose is natural sugar and does not have a toxic effect on the cryopreserved tissue at room temperature; therefore, it need not be removed from the previously cryopreserved adipose tissue after thawing, before the tissue is administered in vivo. Trehalose serving as a CPA can potentially reduce the time after thawing by omitting

Figure 3. Routine histology of the fresh or cryopreserved adipose aspirates (hematoxin–eosin stain; magnification: ×200) showed the normal structure of fragmental adipose tissue from the fresh control group (A) and an adequately maintained, near-normal structure of adipose tissue after optimal cryopreservation of adipose aspirates when the concentration of trehalose was 0.35 mol/L (B).
two or three centrifugations and avoiding osmotic stress to living adipocytes after cryopreservation. Therefore, at least in theory, the use of trehalose as a CPA with its optimal concentration may adequately maintain the viability of cryopreserved adipose tissue after thawing. Further in vivo studies will be conducted to confirm our findings.

**CONCLUSIONS**

The results from the present study show the potential use of trehalose as a CPA for the long-term preservation of adipose aspirates by means of a modern cryopreservation approach. Trehalose, at a concentration of 0.35 mol/L, may be an effective CPA that appears to provide adequate protection of adipose tissue during cryopreservation. Further in vivo studies are needed to confirm these results.

**DISCLOSURES**

The authors have no financial interest in and receive no compensation from manufacturers of products mentioned in this article.

**REFERENCES**