The Effect of Lipoaspirates Cryopreservation on Adipose-Derived Stem Cells

Wei Z. Wang, MD; Xin-Hua Fang, MT; Shelley J. Williams, MT; Linda L. Stephenson, MT; Richard C. Baynosa, MD; Nancy Wong, MD; Kayvan T. Khiabani, MD; and William A. Zamboni, MD

Abstract

Background: Autologous fat grafting has gained popularity, particularly with the discovery of adipose-derived stem cells (ADSC). The possibility of freezing lipoaspirates (LA) for later use has intriguing clinical potential. However, the effect of LA cryopreservation on ADSC is unclear.

Objectives: The authors explore the effect of LA cryopreservation on ADSC viability.

Methods: Human LA (n = 8) were harvested using a standard technique. Lipoaspirate samples were either processed immediately as fresh LA (A) or stored at −20°C and then at −80°C for 30 days with (B) or without (C) freezing medium. Stromal vascular fraction (SVF) was separated from adipocytes and either cultured to obtain purified ADSC or processed for the isolation of 3 distinct ADSC subpopulations (CD90+/CD45–, CD105+/CD45–, and CD34+/CD31–). Apoptosis and necrosis were determined by an annexin V/propidium iodide assay and quantified by flow cytometry. The capability of ADSC for long-term proliferation and differentiation was also examined.

Results: There were no significant differences in the apoptosis and necrosis of adipocytes, SVF, or ADSC between groups A and B. However, cell viability in SVF and ADSC was significantly compromised in group C as compared with group B (P < .01) due to higher ADSC apoptosis but not necrosis. The viable ADSC isolated from fresh or frozen LA were cultured for more than 20 passages and demonstrated similar patterns and speed of proliferation with strong capability to differentiate, evidenced by cell doubling time and positive staining with Oil Red O (Sigma-Aldrich, St Louis, Missouri) and alkaline phosphatase.

Conclusions: Lipoaspirates cryopreservation had a significant impact on ADSC apoptosis but not on ADSC necrosis, proliferation, or differentiations. Freezing medium provides significant protection against ADSC apoptosis.

Keywords
cryopreservation, apoptosis, necrosis, adipocytes, adipose-derived stem cells, SVF, fat grafting, lipoaspirates

Accepted for publication December 19, 2012.

Currently, fat grafting procedures must be conducted immediately after liposuction because the cell death rate in the lipoaspirates (LA) could increase significantly 24 hours after liposuction.1 Due to frequent graft volume loss, some patients require multiple procedures of fat grafting. Therefore, it has been a strong desire of both surgeons and patients to find a method for cryopreserving LA for potential future applications,2 with the advantage being that fat harvesting need only be performed once; thereafter, outpatient fat grafting procedures can be performed using cryopreserved LA.

Although adipose-derived stem cells (ADSC) are a minor fraction of LA and make up less than 1% of the tissue volume,3 investigators have been encouraged by ADSC due to their high capacity for proliferation and differentiation, which might compensate for graft volume loss. Recently, cell-assisted fat grafting4 has gained popularity in the field of plastic surgery. However, the effect of LA cryopreservation on ADSC is unclear and controversial. For example, some investigators5,6 reported that ADSC’s
morphology, proliferation, and differentiation were all significantly hampered by the freeze-thaw process, but others suggested that cryopreservation had no significant impact on ADSC's viability, proliferation, and differentiation.

In this study, we sought to clarify whether cryopreservation of fresh human LA could have a significant impact on ADSC viability, long-term proliferation, and differentiation and whether a freezing medium containing certain cryoprotective agents is needed for LA cryopreservation. We employed the annexin V/propidium iodide (PI) assay (Becton Dickinson, San Jose, California) with the aid of flow cytometry to examine the percentage of live, apoptotic, or necrotic adipocytes; stromal vascular fraction (SVF); and ADSC in thawed LA as compared with fresh LA. ADSC's capability for long-term proliferation and differentiation was examined by cell doubling time and staining of Oil Red O (Sigma-Aldrich, St Louis, Missouri) and alkaline phosphatase.

METHODS

The human LA were harvested from 8 adults who underwent outpatient, cosmetic liposuction procedures. All participants provided informed consent and agreed to inclusion in the study. The Institutional Review Board of the University of Nevada, Reno approved all protocols involving human tissue and cells. Lipoaspirates were harvested by standard liposuction techniques and by a single senior plastic surgeon (W.A.Z.). In brief, through a 4-mm incision, wetting solution (1000 mL of Ringer's lactate containing 30 mL of 1% lidocaine plus 1 mg epinephrine, resulting in a 1:1000 000 concentration of epinephrine) was infiltrated into the subcutaneous fat at a ratio of 1:1 (infiltrate volume vs aspirate volume). The lipoaspirate was procured using a syringe technique with a blunt-tipped 3.7-mm mercedes catheter.

LA Freezing and Thawing

Each LA sample was divided into 2 or 3 portions (A, B, or C) based on the volume of each LA sample obtained. Portion A was processed immediately as fresh LA and served as a control. Portion B was added to an equal volume of freezing medium, which contained nonhematopoietic expansion medium (NHEM; Miltenyi Biotec, Auburn, California) with 10% fetal bovine serum (FBS), 10% dimethyl sulfoxide (DMSO), and 1% penicillin-streptomycin. Nothing was added to portion C. Both portions B and C were stored at −20°C for the first 2 hours and then moved to an −80°C freezer for 30 days of storage. Frozen LA samples (B and C) were quickly thawed in a water bath at 37°C after 30 days of cryopreservation.

Isolation of Adipocytes and SVF

The method for isolation of adipocytes and SVF was described in our previous publication. Briefly, LA samples (either fresh or thawed) were washed with phosphate-buffered saline (PBS) and then centrifuged at 430 g for 10 minutes. After oil was removed, the lipid phase of the lipoaspirate from the top of the conical tube was harvested and diluted with an equal volume of collagenase digestion solution (final concentration: 0.3 U/mL, Collagenase NB 4G proved grade; Serva Electrophoresis, Heidelberg, Germany). After 30 minutes of incubation, an equal volume of Dulbecco’s modified Eagle’s medium containing 20% FBS was added to stop enzymatic digestion. The floating layer containing adipocytes and the pellet containing SVF were separated by centrifugation. The isolated adipocytes were then passed through a 100-µm cell strainer, and the total number of adipocytes was counted. The isolated SVF was filtered sequentially through a 100-µm nylon filter, followed by a 30-µm filter, and then processed for density gradient by centrifugation with Histopaque-1077 (Sigma-Aldrich). The white band (mononuclear cells) remaining at the plasma interface was carefully aspirated, and the total number of SVF cells was counted. Harvested SVF was then either cultured to obtain purified ADSC or processed for the isolation of 3 distinct ADSC subpopulations (CD90+/CD45−, CD105+/CD45−, and CD34+/CD31−).

Purification of ADSC Through SVF Culture

The SVF is highly heterogeneous and contains many cell subsets, including ADSC, endothelial cells, hematopoietic cells, and others. One of the characteristics of ADSC is that they are adherent to the plastic surface. Therefore, isolation of ADSC can be achieved through SVF culture. The method for SVF culture has been described in our previous publication. In brief, 1 × 10^6 of SVF cells and 15 mL of pre-warmed NHEM containing 1% penicillin-streptomycin were added into a 75-cm² cell culture flask, respectively, and cultivated at 37°C, 5% CO₂, and 95% humidity. After 24 hours of culturing, the nonadherent cells in the flask were removed by PBS washing. Fresh prewarmed NHEM (15 mL) was added to the flask for continuation of culture. Cell adhesion was examined under an inverted microscope. The cultured ADSC were checked daily and the medium was changed every 3 days. Passageing was performed every 6 days by removing the old medium, washing PBS, adding 1 mL of trypsin/EDTA, and incubating at 37°C for 10 minutes. After complete dissociation, the cells were washed by fresh NHEM and then split up for subculture. To continue culturing, 1.25 × 10^5 of ADSC were transferred to a new T-25 flask with 5 mL of fresh NHEM. Cell number was counted on each passage to calculate cell doubling time.
Adipocyte Differentiation and Detection

The method for adipocyte differentiation and detection was described in our previous publication. Briefly, 1 × 10^5 of ADSC were diluted in 2 mL of prewarmed Nonhematopoietic AdipoDiff Medium (NHAM; Miltenyi Biotec) or 2 mL of NHEM, which served as a control. Part of the cell suspension (1.5 mL) was then transferred to a 35-mm cell culture dish and cultivated at 37°C, 5% CO_2, and 95% humidity. The media were changed every 3 days. On day 21, cells were analyzed by Oil Red O staining for adipocyte detection. Culture dishes were washed by Dulbecco’s PBS (DPBS), and 2 mL of 10% formalin was added and incubated for 60 minutes. After formalin was removed, 2 mL of 60% isopropanol was added. Isopropanol was removed and 2 mL of the working solution (prepared by Oil Red O powder and 99% of isopropanol) was added to the dish. Cells were then washed with 2 mL of Harris hematoxylin solution. Finally, hematoxylin was removed and cells were observed on an inverted light microscope.

Osteoblast Differentiation and Detection

The method for osteoblast differentiation and detection was described in our previous publication. Briefly, 6 × 10^4 of ADSC were diluted in 2 mL of prewarmed Nonhematopoietic OsteoDiff Medium (NHODM; Miltenyi Biotec) or 2 mL of NHEM, which served as a control. Part of the cell suspension (1.5 mL) was then transferred to a 35-mm cell culture dish and cultivated at 37°C, 5% CO_2, and 95% humidity. The media were changed every 3 days. On day 10, cells were stained by alkaline phosphatase for osteoblast detection. After the medium was removed, precooled methanol (2 mL) was added and incubated for 5 minutes at −20°C. Methanol was then removed and SIGMAFAST BCIP/NBT substrate (2 mL; Sigma-Aldrich) was added to the dish and agitated on a plate shaker for 10 minutes. As alkaline phosphatase expressed by osteoblasts processed the substrate, the cells stained dark purple. Finally, cells were washed and examined under a microscope.

Detecting ADSC Subpopulations From SVF

The method for detecting 3 distinct ADSC subpopulations, including CD90^+CD45^−, CD105^+CD45^−, and CD34^+CD31^−, was described in our previous study. Briefly, 1 × 10^5 SVF cells were stained by 5 µL CD90-PE-cy7, 5 µL CD105-PerCP, 20 µL CD34-APC, and 20 µL CD45-PE or CD31-PE and incubated at 2°C to 8°C for 30 minutes. The stained ADSC were then resuspended in 100 µL of binding buffer and incubated with 10 µL of annexin V–FITC. After incubation and centrifugation, the supernatant was removed and 500 µL of binding buffer and 5 µL of PI solution were added. Three tubes were used to set up compensation and quadrants with (1) unstained cells, (2) cells stained with 5 colors (CD90-PE-cy7, CD105-PerCP, CD45-PE, annexin V–FITC, and PI), and (3) cells stained with 4 colors (CD34-APC, CD31-PE, annexin V–FITC, and PI). Necrosis was determined by PI and apoptosis was determined by annexin V–FITC. Ten thousand SVF cells from each sample were scanned. Data acquisition and analysis were performed by a flow cytometer with BD FACSDiva software v6.1.3 (Becton Dickinson, San Jose, California), using an excitation wavelength of 488 nm with an argon laser.

Statistics

All measurements were compared with an analysis of variance test followed by a t test. *P* ≤ .05 was considered significant.

RESULTS

The mean (SEM) age of the participants was 52 (3.8) years with a mean (SEM) body mass index (BMI) of 25 (1.6). Liposuction was performed in the hip (n = 6) or abdomen (n = 2). The mean (SEM) volume of lipoaspirate was 107 (7.2) mL.

In adipocytes, we found that nearly 80% of adipocytes were live, 20% were apoptotic, and 1% were necrotic. There was no significant difference among the 3 groups (Figure 1). In ADSC subpopulations, there was no statistically significant difference in apoptosis and necrosis between groups A and B. However, live ADSC (Figure 2) were significantly lower (P < .01) in all 3 ADSC subpopulations in group C as compared with those in groups A and B due to a significantly higher percentage (P < .01) of ADSC apoptosis (Figures 3 and 4) but not necrosis (Figure 5) in group C. Moreover, CD34^+/31^− ADSC had significantly

Detection of Apoptotic and Necrotic Adipocyte, SVF, and ADSC Subpopulations

The method for apoptotic and necrotic cell staining and flow cytometry analysis was described in our previous studies. Briefly, 1 × 10^5 adipocytes were suspended in 100 µL of binding buffer and incubated with 10 µL of annexin V–FITC. After incubation and centrifugation, the supernatant was removed and 500 µL of binding buffer and 5 µL of PI solution were added. Ten thousand adipocytes from each sample were scanned and analyzed by a flow cytometer. For the detection of apoptotic and necrotic ADSC subpopulations, 1 × 10^5 SVF cells were stained by 5 µL CD90-PE-cy7, 5 µL CD105-PerCP, 20 µL CD34-APC, and 20 µL CD45-PE or CD31-PE and incubated at 2°C to 8°C for 30 minutes. The stained ADSC were then resuspended in 100 µL of binding buffer and incubated with 10 µL of annexin V–FITC. After incubation and centrifugation, the supernatant was removed and 500 µL of binding buffer and 5 µL of PI solution were added. Three tubes were used to set up compensation and quadrants with (1) unstained cells, (2) cells stained with 5 colors (CD90-PE-cy7, CD105-PerCP, CD45-PE, annexin V–FITC, and PI), and (3) cells stained with 4 colors (CD34-APC, CD31-PE, annexin V–FITC, and PI). Necrosis was determined by PI and apoptosis was determined by annexin V–FITC. Ten thousand SVF cells from each sample were scanned. Data acquisition and analysis were performed by a flow cytometer with BD FACSDiva software v6.1.3 (Becton Dickinson, San Jose, California), using an excitation wavelength of 488 nm with an argon laser.
lower rates of apoptosis and necrosis ($P < .01$) compared with CD105+/45– ADSC in all 3 groups.

The ADSC isolated from both fresh LA and frozen LA were subcultured for more than 20 passages and demonstrated rapid long-term proliferation (Figure 6), evidenced by cell doubling time (Figure 7), which ranged from 40 to 80 hours in the first 16 passages. There was no significant difference in the pattern or speed of ADSC proliferation between fresh LA and frozen LA. The ADSC differentiation was examined at passages 3 and 20. The ADSC isolated from both fresh and frozen LA showed adipocyte differentiation with positive staining of Oil Red O (Figure 8) and osteoblast differentiation with positive staining of alkaline phosphatase (Figure 9), even after 20 passages. Moreover, despite a higher percentage of ADSC apoptosis detected in group C, the viable ADSC still showed rapid long-term proliferation and differentiation.

**DISCUSSION**

The effect of LA cryopreservation on adipocytes has been studied for years. Some investigators preferred a slow cooling protocol (1-2°C/min to −30°C and subsequently stored in an ultralow freezer or liquid nitrogen) recommended by Pu et al., while others chose direct freezing in an ultralow freezer or liquid nitrogen. Although ADSC are a minor fraction in the lipoaspirate, they have great potential for regeneration that may compensate for graft volume loss. It is important to know the outcome of the freezing process on ADSC, since a majority of previous studies have examined only adipocytes. The freezing protocol used in the present study (storage in −20°C freezer for the first 2 hours and then in an −80°C freezer) is similar to the slow cooling protocol. In our pilot study, we tested the temperature changes with time with a thermometer stored in the −20°C freezer. We found that the temperature was decreased from +20°C (room temperature) to −5°C within the first 15 minutes, further decreased from −5°C to −15°C in the second 15 minutes, and then decreased 1°C for every 15 minutes after. Obviously, the speed of temperature reduction in a −20°C freezer is similar to the slow cooling protocol. In our pilot study, we tested the temperature changes with time with a thermometer stored in the −20°C freezer. We found that the temperature was decreased from +20°C (room temperature) to −5°C within the first 15 minutes, further decreased from −5°C to −15°C in the second 15 minutes, and then decreased 1°C for every 15 minutes after. Obviously, the speed of temperature reduction in a −20°C freezer is similar to the slow cooling protocol. Importantly, we found that the readings of ADSC apoptosis and necrosis in thawed samples using our freezing protocol and medium were very similar to those seen in the fresh samples.

A number of cryoprotective agents (CPA) have been examined in the past, including DMSO, trehalose, hydroxyethyl starch, glycerol, dextran, sucrose, polyvinyl pyridone, FBS, and serum free. However, there is no universal agreement on this issue. For example, Cui et al. found that a combination of DMSO and trehalose provided good long-term preservation of adipose
Figure 4. A representative illustration of a flow cytometer analysis to explain how the apoptosis and necrosis in each adipose-derived stem cell (ADSC) subpopulation were determined. (A) Unstained sample. (B) Sample stained by CD90 and CD45. (C) Unstained sample stained by annexin V–FITC and propidium iodide (PI). (D) Gated CD90−/CD45− population stained by Annex V–FITC and PI. (E) Gated CD90+/CD45− population stained by annexin V–FITC and PI. SVF, stromal vascular fraction.
aspirates. However, Grewal et al. reported that freezing adipose graft with PBS or various concentrations of FBS and DMSO resulted in morphological changes in vivo and affected their ability to synthesize vascular endothelial growth factor. The freezing medium used in the present study was purchased from a well-known international biotech company (Miltenyi Biotec) that is a pioneer in cell separation technology. In our pilot study, we found that samples frozen with that medium had a lower ADSC apoptosis than media in which FBS or DMSO was not included. Our result is corroborated by the report from Lee et al., who found that ADSC isolated from thawed LA that was cryopreserved with 10% FBS and 10% DMSO could survive and differentiate into adipocytes in vitro. No ADSC was detected in thawed LA samples that were frozen without DMSO.

The effect of cryopreservation on cultured ADSC is also controversial. For example, James et al. reported that ADSC’s cellular morphology, proliferation, and osteogenic and adipogenic differentiation were all significantly hampered by the freeze-thaw process after 2 weeks of ADSC cryopreservation with 90% FBS and 10% DMSO. On the other hand, Gonda et al. reported that ADSC could be cryopreserved with 10% FBS for up to 6 months without any loss of proliferative or differentiation potential. Thirumala et al. reported that the thawed ADSC viability and adipogenic and osteogenic differentiability could be maintained even when they were frozen in the absence of serum but with 2% DMSO or 10% polyvinylpyrrolidone. The absence of DMSO significantly increases the fraction of apoptosis and necrosis of ADSC. Moreover, De Rosa et al. and Liu et al. separately reported that ADSC cryopreservation had no negative effect on the phenotype, proliferation, or osteogenic differentiation of human ADSC.

One of the potential reasons for conflicting reports is differences in the freezing medium or CPA used in cryopreservation. For example, James et al. used a high concentration of serum (90% FBS), Lee et al. used a low concentration of serum (10% FBS), and Thirumala et al. used no serum. Different cell viability assays could be another potential reason for conflicting results. For example, some investigators used a trypan blue assay to determine ADSC viability. However, trypan blue can only determine cell necrosis based on cell membrane rupture; it cannot identify apoptotic cells with an intact cell membrane. Current literature has indicated that necrosis is no longer considered the sole mechanism of cell death, and apoptosis might be the initial mode of cell death in the process to ultimate cell death.

Adipose-derived stem cells are a group of cells that have various subpopulations, and each subpopulation of ADSC has unique surface antigens or phenotypes. For example,
Figure 8. (A) Control sample for Oil Red O staining of adipose-derived stem cells (ADSC) (P20) isolated from frozen lipoaspirates (LA). (B-E) Experimental samples for Oil Red O staining of ADSC (P20) isolated from frozen LA.
CD90+ and CD105+ cells are defined as mesenchymal stem cells (MSC) based on the International Society for Cellular Therapy (ISCT) statement of minimal criteria for defining MSC.30,31 Although CD34+ cells are not included in ISCT criteria, a number of reports32-34 have shown that ADSC isolated from human lipoaspirate are routinely CD34+, although loss of CD34+ expression in culture is well documented.35-37 Although ADSC have become some of the most studied adult stem cells, isolation of pure ADSC from SVF is difficult because ADSC lack a specific cell surface marker. In our previous study,14 a panel of stem cell markers was used for staining the cultured human ADSC. We found that 87% of human cultured ADSC were CD90+, 61% were CD105+, 17% were CD34+, and 7% were CD271+. All ADSCs were negative on CD45 or CD31. To obtain relatively purified ADSC from SVF, we selected the most positive markers together with the most negative markers to detect the 3 most popular ADSC subpopulations (CD90+/CD45−, CD105+/CD45−, and CD34+/CD31−). However, it is possible that some ADSC could have more than 1 phenotype or overlap. If an ADSC has both positive CD90+ and CD34+, that particular cell should be defined as CD90+ and CD34+ ADSC as long as it is negative on CD45 or CD31.

Many factors (such as BMI, age, sex, medical conditions, liposuction location, and liposuction technique) could affect the results. However, the influence of these factors has been largely diminished or controlled by our experimental design. In the present study, each lipoaspirate sample was divided into 3 portions (A: fresh, B: frozen with medium, and C: frozen without medium). If a patient with a lower BMI has higher CD34+ ADSC, all 3 portions should have higher CD34+ ADSC. In this experimental design, the difference between groups A and C is the freezing process. The difference between B and C groups is the freezing medium.

All data from our study are quantitative except the pictures of Oil Red O and alkaline phosphatase staining. Therefore, we can tell that some ADSC were still able to differentiate even after P20, but we could not determine whether there was a difference in the differentiation rate between P3 and P20 ADSC. For a future study, we will select a quantitative method to measure ADSC differentiation.

In summary, in the present study, fresh human LA were cryopreserved with or without freezing medium for 30 days. The ADSC were isolated from thawed LA and then evaluated by apoptosis, necrosis, long-term proliferation, and differentiation. Our results can be summarized as follows: (1) LA cryopreservation had no effect on adipocyte viability but had a significant impact on ADSC apoptosis. (2) The freezing medium (containing 10% FBS and 10% DMSO) provided great protection against ADSC apoptosis. (3) CD34+/CD31− ADSC had significantly lower rates of apoptosis and necrosis compared with those in CD105+/CD45− ADSC, but that phenomenon has nothing to do with freezing medium and cryopreservation. (4) The viable ADSC isolated from fresh or frozen LA showed similar patterns and speed of proliferation. Even after 20 passages, ADSC still showed differentiation capability.

CONCLUSIONS

Developing effective methods for the cryopreservation of human LA could reduce the cost and complications of liposuction procedures. In the present study, we focused on the effect of LA cryopreservation on ADSC. Although

Figure 9. (A) Control sample for alkaline phosphatase staining of adipose-derived stem cells (ADSC) (P20) isolated from frozen lipoaspirates (LA). (B) Experimental sample for alkaline phosphatase staining of ADSC (P20) isolated from frozen LA.
the ADSC are a minor fraction and make up less than 1% of the adipose volume, they have great potential for regeneration that may compensate for graft volume loss if we are able to control ADSC proliferation and differentiation in vivo. Our results indicate that the freezing medium we used can provide significant protection against ADSC apoptosis produced by the freezing process. Further investigation is needed to determine which factor in the freezing medium is the major contributor for ADSC protection.

Disclosures

The author declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Funding

The author received no financial support for the research, authorship, and publication of this article.

REFERENCES


