Influences of Preservation at Various Temperatures on Liposuction Aspirates

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Background: Aspirated fat is not only a filler material but also an abundant source of adipose-derived stem cells. The aim of this study was to assess degeneration of aspirated fat during preservation and optimize the preservation method for lipoaspirates.
Methods: Aspirated fat was preserved at room temperature for 1, 2, 4, and 24 hours ($n = 10$ each); at 4°C for 1, 2, and 3 days ($n = 14$ each); or at −80°C for 1 month ($n = 3$). Morphologic changes were assessed with scanning electron microscopy. Adipose-derived stem cell yield was measured after 1 week of culture. For aspirated fat preserved at room temperature, damaged adipocytes were assessed by measuring the oil volume ratio after centrifugation ($n = 6$) and glycerol-3-phosphate-dehydrogenase activity in washing solution ($n = 4$). Cell surface marker expression was examined by flow cytometry ($n = 3$).
Results: Although the scanning electron microscopic assay indicated no remarkable anatomical changes based on preservation methods, oil volume significantly increased in fat preserved at room temperature for 4 hours. Adipose-derived stem cell yield was significantly reduced by preservation at room temperature for 24 hours and by preservation at 4°C for 2 or 3 days. Flow cytometric analysis suggested that the biological properties of adipose-derived stem cells did not significantly change at 4°C up to 3 days. The cells were isolated from cryopreserved fat, but the yield was much less than that from fresh aspirated fat.
Conclusions: Aspirated fat should be transplanted as quickly as possible if it is preserved at room temperature. For adipose-derived stem cell isolation, aspirated fat can be stored or transported overnight if it is preserved at 4°C without adipose-derived stem cell yield loss or changes in biological properties. (*Plast. Reconstr. Surg.* 120: 1510, 2007.)

Although fat tissue has been used as a filler material for more than 100 years,[1,2] there are several problems to be resolved, including unpredictability and fat necrosis resulting in infection and calcification.[2,3] Autologous fat transfer, however, is almost the only method of soft-tissue augmentation that can be performed without detectable scarring on either a donor or a recipient site and without complications associated with foreign materials. Because fat tissue is more easily damaged by ischemia compared with other tissues such as skin and bone, transferring fat tissue as quickly as possible after harvesting is recommended. Thus, it is of great interest to investigate how adipose tissue viability after aspiration changes over time depending on preservation methods. Clinically, aspirated fat tissue is usually preserved at room temperature in a suction bottle, but how aspirated fat in the suction bottle changes during the postoperative hours is unknown.

It was recently revealed that adipose tissue is a remarkable source of multipotent stem cells,[4,5] which can differentiate into adipogenic, chondrogenic, osteogenic, myogenic, neurogenic, endothelial, and other lineages.[6] Adipose-derived stem cells have already been used in some clinical trials, including treatments for bone defects[7]; rectovaginal fistula[8]; and soft-tissue augmentation, including breast enhancement, breast reconstruction, and facial rejuvenation.[9] Adipose-derived stem cells may be clinically used or banked also for other therapeutic purposes in

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the near future. In practice, there is some time lag between liposuction and cell isolation; it takes a few to several hours for liposuction surgery, depending on the volume and sites to suction, and a few hours to even a day or two for transportation from an operating room to a cell-processing unit. To maximize the potential of adipose aspirates as a stem cell source, it is very important to optimize protocols for their preservation.

Thus, aspirated fat is now valuable as an autologous filler material and as an abundant stem cell source. We sought to comprehensively evaluate the influences of preservation at different temperatures on the viability of aspirated fat and adipose-derived stem cells.

PATIENTS AND METHODS

Human Tissue Sampling and Preservation

We obtained liposuction aspirates from 21 healthy female donors undergoing liposuction of the abdomen or thighs after informed consent using an institutional review board–approved protocol. The adipose portion of the liposuction aspirates was preserved at room temperature for 1, 2, 4, or 24 hours ($n = 10$); at 4°C for 0, 1, 2, and 3 days ($n = 14$); or at −80°C for 1 month ($n = 3$) and subjected to assays as described below. Excised fat obtained from a cosmetic surgery patient was also used for comparison.

Cell Processing and Culture

Stromal vascular fractions were isolated from the fatty portion of liposuction aspirates as previously described.[10] Briefly, the aspirated fat was washed with phosphate-buffered saline and digested on a shaker at 37°C in phosphate-buffered saline containing 0.075% collagenase for 30 minutes. Mature adipocytes and connective tissues were separated from pellets by centrifugation (800 *g* for 10 minutes). The cell pellets were resuspended, filtered with a 100-μm mesh (Millipore, Bedford, Mass.), plated (30,000 cells/cm^2) onto gelatin-coated dishes, and cultured at 37°C in an atmosphere of 5% carbon dioxide in humid air. The culture medium was M-199 containing 10% fetal bovine serum, 100 IU of penicillin, 100 mg/ml streptomycin, 5 μg/ml heparin, and 2 ng/ml acidic fibroblast growth factor. Medium was replaced every third day. After 7 days, adherent cells were trypsinized and counted with a cell counter (NucleoCounter; ChemoMetec, Allerod, Denmark).

Flow Cytometric Analysis

Adherent adipose-derived stem cells were examined for surface marker expression using flow cytometry after 1 week of culture ($n = 3$). The following monoclonal antibodies were used: CD29-PE; CD31-PE; CD34-PE; CD45-PE; CD90-PE; CD133-PE; CD144-PE; HLA-A, B, and C-PE; Tie-2-PE (BD Biosciences, San Diego, Calif.); CD105-PE (Serotec, Oxford, United Kingdom); and Flk-1-PE (Techne, Princeton, N.J.). Cells were incubated with the directly conjugated monoclonal antibodies in phosphate-buffered saline containing 0.5% bovine serum albumin for 30 minutes at 4°C, washed with phosphate-buffered saline containing 0.2% bovine serum albumin, and diluted in phosphate-buffered saline containing 0.1% bovine serum albumin. Flow cytometric analyses were performed using an LSR2 (Becton Dickinson, San Jose, Calif.).

Quantitative Analysis of Damaged Adipocytes in Aspirated Fat

To assess damaged adipocytes in aspirated fat, we measured the ratios of oil and fat volumes after centrifugation ($n = 6$), and an extracellular activity of an enzyme, glycerol-3-phosphate-dehydrogenase, with a GPDH Activity Measurement Kit (Cell Garage, Tokyo, Japan) ($n = 4$). The enzyme is normally strictly intracellular, and the enzyme activity in a washing solution is proportional to the amount of adipocyte destruction.

For measurement of the oil ratio, the fatty portion of liposuction aspirates was divided into 20 tubes (15-ml conical tubes) and preserved at room temperature. After preservation for 1, 2, 4, or 24 hours, five of the tubes were centrifuged at 2330 *g* for 5 minutes to separate the oil, fat, and fluid into distinct layers from top to bottom (Fig. 1). The oil ratio was calculated as follows: oil ratio = (oil volume)/[(oil volume) + (fat volume)]. Data were collected from lipoaspirates obtained from six patients.

For extracellular glycerol-3-phosphate-dehydrogenase assessment, after 1 g of aspirated fat lobules was washed in a tube with 4 ml of phosphate-buffered saline, 1 ml of the phosphate-buffered saline solution was transferred to another tube and centrifuged at 15,000 *g* for 5 minutes. After the supernatant was appropriately (two to 20 times) diluted with the enzyme extracting reagent, 100 μl of each diluted sample was transferred onto a 96-well plate. The enzyme reaction was then started by the addition of 200 μl of substrate reagent containing dihydroxyacetone phosphate

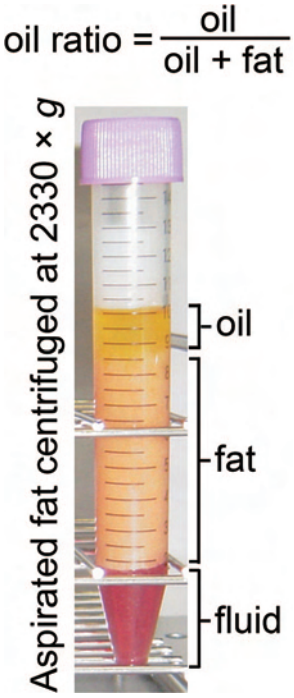

Fig. 1. Analysis of adipocyte damage in lipoaspirates by centrifugation. After centrifugation, aspirated fat tissue was separated into distinct layers from top to bottom: the oil, fat, and fluid layers. Adipocyte damage by preservation was quantified by calculating the oil ratio in the volume as follows: *oil ratio* = (*oil volume*)/[(*oil volume*) + (*fat volume*)].

and nicotinamide adenine dinucleotide (NADH). The assay measures the reduction of NADH to NAD+ in the presence of glycerol-3-phosphate-dehydrogenase. The absorbance at 340 nm was recorded every 30 seconds for 10 minutes by using a spectrometer (DTX880; Beckman Coulter, Fullerton, Calif.) to produce an extinction curve for calculating the maximum decrease, indicating the glycerol-3-phosphate-dehydrogenase activity.

Procedure for Cryopreserving and Thawing Aspirated Fat

Fresh adipose tissue was mixed with an equal amount of freezing medium (Cell Banker BLC-1; ZENOAQ, Fukushima, Japan) and cooled to −80°C in a programmable freezing system (TNP-87S; Nihon Freezer, Tokyo, Japan) at a freezing rate of 1°C every 15 minutes. After 1 month, cryopreserved adipose tissue was thawed by placement of the cryotube into a water bath at 37°C.

Scanning Electron Microscopic Study

After preservation at room temperature or 4°C, aspirated fat was fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M cacodylate buffer for 1 week at room temperature and then fixed in 1% osmium tetroxide. After dehydration, samples were dried with a supercritical point carbon dioxide dryer (HCP-2; Hitachi, Tokyo, Japan), sputter-coated with platinum/palladium, and examined with a scanning electron microscope (S3500N; Hitachi).

Statistical Analysis

Results were expressed as mean ± SE. Paired or unpaired *t* tests were performed to evaluate differences between groups, and no correction was made for multiple comparisons.

RESULTS

Morphology of Adipocytes in Aspirated Fat

Aspirated fat was preserved at 4°C and fixed for scanning electron microscopic study at different time points. Adipocytes from aspirated fat almost retained their round shape and showed no significant morphologic differences compared with those of excised fat (Fig. 2, *above, left*). No remarkable change in adipocyte morphology was found in aspirated fat tissues on days 0, 1, and 3 (Fig. 2, *above, second from left, second from right,* and *right*).

Aspirated fat was preserved for 1, 2, 4, or 24 hours at room temperature and evaluated by scanning electron microscopy also (Fig. 2, *below*). No remarkable difference in adipocyte morphology was identified.

Degeneration of Adipocytes with Preservation Time

Because we clinically observe a gradual increase of oil volume in lipoaspirates, the oil ratio (oil volume/[oil volume + fat volume]) was used as one of indices of degeneration of adipocytes in aspirated fat. The oil ratio increased over time during preservation at room temperature (Fig. 3, *above, left*). Oil ratios at 4 and 24 hours were significantly greater than those at 1 hour, and the oil ratio per-hour increase slowed remarkably after 4 hours (Fig. 3, *above, right*). Glycerol-3-phosphate-dehydrogenase activity in a washing solution of preserved fat increased slightly up to 4 hours and increased markedly after 24 hours of preservation (Fig. 3, *below, left*). Unlike the results with the oil ratio, glycerol-3-phosphate-dehydrogenase activity increase per hour did not change up to 4 hours but significantly increased after that time point.

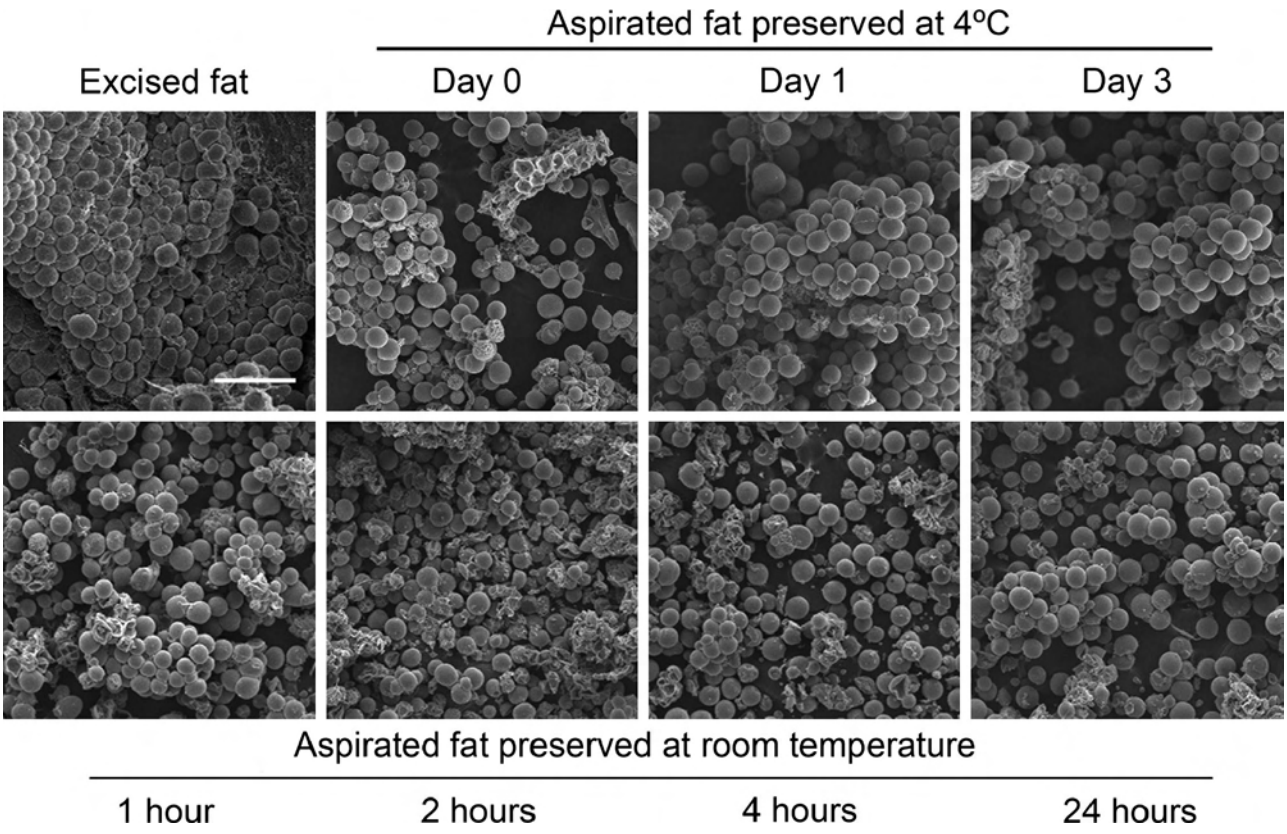

Fig. 2. Comparison with scanning electron microscopy of human aspirated fat tissues after preservation at 4°C or room temperature. (*Above*, *left*) Excised adipose tissue was fixed immediately after the operation. Aspirated fat tissues preserved at 4°C were fixed on day 0 (*above*, *second from left*), day 1 (*above*, *second from right*), or day 3 (*above*, *right*), whereas those preserved at room temperature were fixed at 1 hour (*below*, *left*), 2 hours (*below*, *second from left*), 4 hours (*below*, *second from right*), or 24 hours (*below*, *right*) after the operation. Each sample was treated for evaluation with scanning electron microscopy, and representative photographs are shown. No significant morphologic changes over time were found by scanning electron microscopy in aspirated fat, even in samples stored at 4°C for 3 days or at room temperature for 24 hours. *Scale bar* = 250 μm.

Adipose-Derived Stem Cell Yield from Aspirated Fat Preserved at Room or Cool Temperature

When preserved at room temperature, adipose-derived stem cell yield was maintained up to 4 hours and decreased remarkably at 24 hours (Fig. 4). In contrast, after preservation at 4°C, almost the same number of adipose-derived stem cells were isolated from aspirated adipose tissue on days 0 and 1 (Fig. 5). The number of isolated adipose-derived stem cells was extensively decreased in some cases on day 2 and in all cases on day 3. Statistical significance was seen between days 0 and 3 ($p < 0.001$) and between days 1 and 3 ($p < 0.001$).

Surface Marker Expression of Adipose-Derived Stem Cells Isolated from Aspirated Fat Preserved at a Cool Temperature

To examine changes in the biological properties of adipose-derived stem cells based on preservation time at a cool temperature, surface marker analysis was performed on adipose-derived stem cells isolated from aspirated fat tissues preserved for 0, 1, 2, and 3 days at 4°C (Table 1).

Adipose-Derived Stem Cell Yield from Cryopreserved Aspirated Fat

We also evaluated the possibility of isolating adipose-derived stem cells from aspirated fat cryopreserved for 30 days ($n = 3$). Adipose-derived stem cells were harvested from the cryopreserved aspirated fat, but the adipose-derived stem cell yield was significantly less than that obtained from fresh aspirated fat (Fig. 6).

DISCUSSION

The scanning electron microscopic assay showed that no significant morphologic change in adipocytes was found among aspirated fat tissues preserved either at 4°C for up to 3 days or at

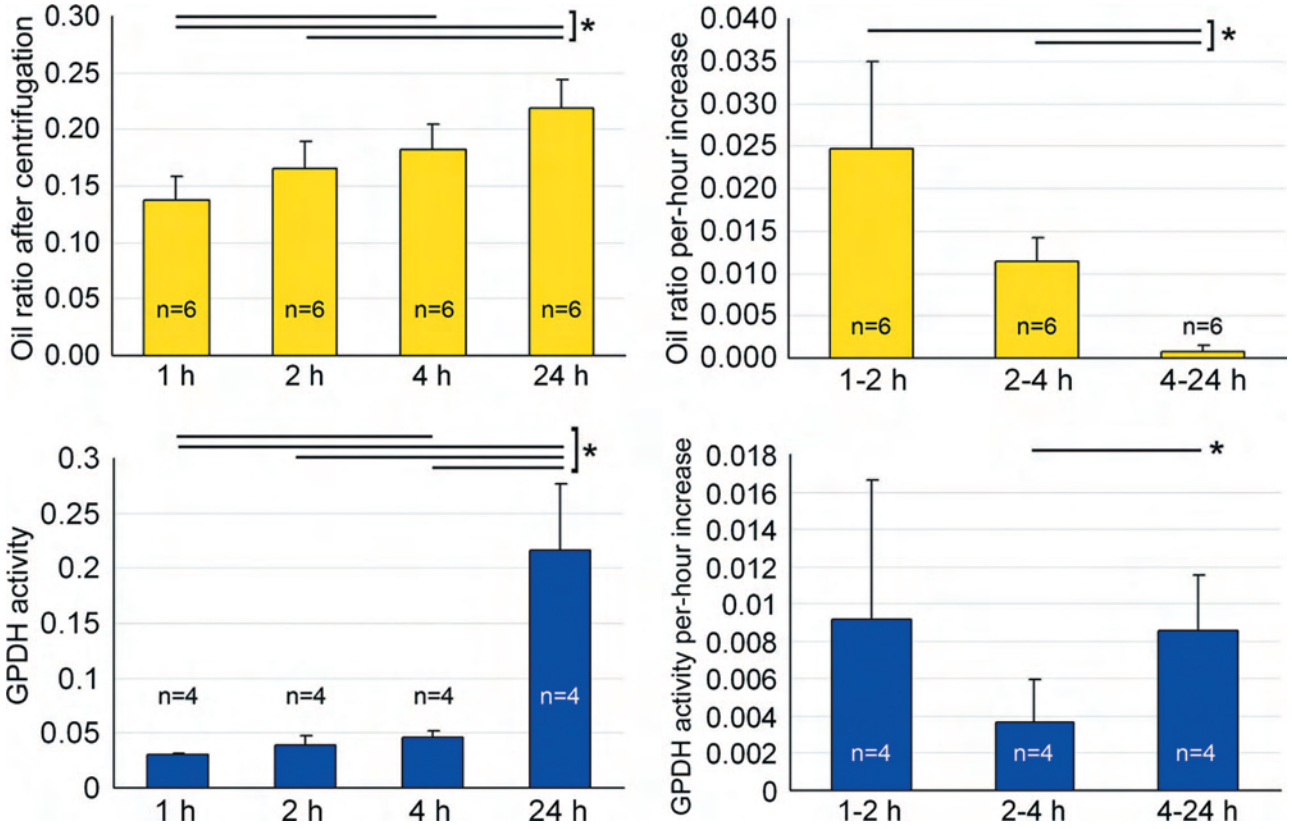

Fig. 3. Assessment of damaged adipocytes after preservation at room temperature. Damaged adipocytes were evaluated by measurement of the oil ratio after centrifugation (*above*) or glycerol-3-phosphate-dehydrogenase activity of preserved fat in washing solution (*below*). Statistical analysis was performed using paired *t* tests between groups. Values are mean ± SE; $*p < 0.05$. (*Above*, *left*) Oil ratios in aspirated fat preserved at room temperature for 1, 2, 4, or 24 hours are shown. The oil volume ratio gradually increased with storage time, likely because of the breakdown of adipocytes. (*Above*, *right*) The oil ratio per-hour increase slowed remarkably with storage time. (*Below*, *left*) Glycerol-3-phosphate-dehydrogenase activity of preserved fat in washing solution for 1, 2, 4, or 24 hours was measured for assessment of damaged adipocytes. Glycerol-3-phosphate-dehydrogenase activity had slightly increased up to 4 hours and was markedly increased at 24 hours. (*Below*, *right*) Glycerol-3-phosphate-dehydrogenase activity increase per hour did not change significantly up to 4 hours, but did increase after 4 hours.

room temperature for up to 24 hours. However, quantitative analysis of damaged adipocytes by measuring the oil ratio and glycerol-3-phosphate-dehydrogenase activity revealed that preserved adipocytes were partly degenerated and ruptured over preservation time when stored at room temperature. Thus, preservation at room temperature resulted in damage to some adipocytes that may have been located superficially; however, the remaining adipocytes retained almost-intact morphology.

In this study, damaged adipocytes were evaluated by measurement of the oil ratio after centrifugation and glycerol-3-phosphate-dehydrogenase activities in washing solution of preserved fat. Boschert et al.[11] reported that centrifugation at greater than 100 *g* caused adipose cell destruction; however, we recently found that centrifugation at 400 *g* increased the oil portion in lipoaspirates but that further centrifugation did not significantly damage adipocytes or increase oil volume.[12] In addition, our histologic examinations of centrifuged aspirated fat with light and scanning electron microscopy showed that adipocytes appeared to be intact even after centrifugation at 4300 *g*.[12] Therefore, we considered that the increased oil volume after centrifugation (for 5 minutes at 2330 *g*) in the present study was attributable to adipocyte damage from preservation at room temperature. The measurement of glycerol-3-phosphate-dehydrogenase activity produced results that were similar to those of the oil ratio up to 4 hours but that differed somewhat at 24 hours; glycerol-3-phosphate-dehydrogenase activity had increased remarkably after 24 hours of preservation. This difference might be attributable to incomplete

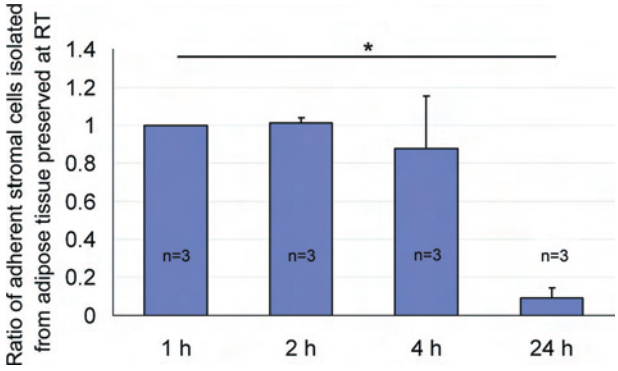

Fig. 4. Adipose-derived stem cell yield after preservation at room temperature. We preserved aspirated adipose tissues at room temperature for 1, 2, 4, or 24 hours and processed them for isolation of adipose-derived stem cells, which were then cultured for 1 week. Ratios of adipose-derived stem cell yield to control (1-hour preservation) were calculated; data were obtained from three patients, and statistical analysis was performed using paired *t* tests between 1-hour and other groups. Adipose-derived stem cell yield seemed to be maintained for up to 4 hours of preservation and decreased remarkably when preserved for 24 hours at room temperature. Values are mean ± SE; $*p < 0.05$.

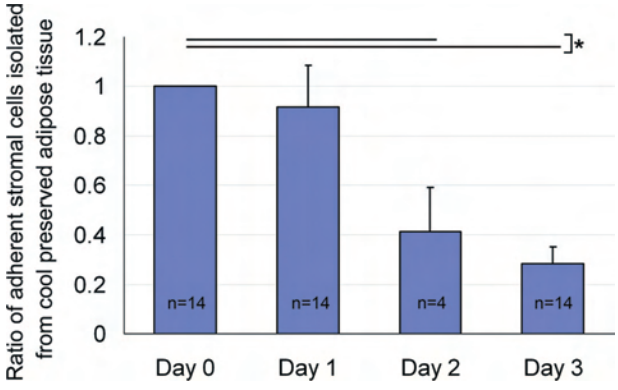

Fig. 5. Adipose-derived stem cell yields after preservation at 4°C. We preserved aspirated fat tissues at 4°C for 0, 1, 2, and 3 days and processed them for isolation of adipose-derived stem cells, which were then cultured for 1 week. Ratios of adipose-derived stem cell yield to control (day 0, no preservation) were calculated; data were obtained from 14 patients (data for day 2 came from four of the 14 patients), and statistical analysis was performed using unpaired *t* tests between day 0 and other groups. A statistical difference in adipose-derived stem cell yield was not found between days 0 and 1, whereas adipose-derived stem cell yield decreased significantly on days 2 and 3. Values are mean ± SE; $*p < 0.05$.

Table 1. Surface Marker Expression of Adipose-Derived Stem Cells Isolated from Aspirated Fat Tissues Preserved at 4°C*

	Day 0	Day 1	Day 2	Day 3
CD29	98.7 ± 0.8	98.4 ± 0.8	99.4 ± 0.1	97.3 ± 1.8
CD31	1.7 ± 0.3	2.0 ± 0.4	1.4 ± 0.8	0.7 ± 0.1
CD34	48.1 ± 6.5	46.3 ± 5.5	35.8 ± 5.2	31.2 ± 3.4
CD45	1.0 ± 0.3	1.2 ± 0.4	0.7 ± 0.2	1.1 ± 0.6
CD90	99.2 ± 0.5	98.1 ± 0.9	99.2 ± 0.1	97.1 ± 2.1
CD105	97.8 ± 1.3	97.0 ± 2.2	97.8 ± 0.8	96.2 ± 2.1
CD133	3.1 ± 1.5	2.2 ± 1.6	0.9 ± 0.6	1.0 ± 0.4
CD144	0.5 ± 0.2	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
HLA-ABC	98.3 ± 0.8	97.4 ± 1.6	96.5 ± 1.6	93.6 ± 3.0
Flk-1	56.1 ± 17.2	54.4 ± 26.5	81.7 ± 4.4	71.0 ± 5.5
Tie-2	2.2 ± 17.2	2.3 ± 0.9	1.7 ± 0.6	1.8 ± 0.4

*Adipose-derived stem cells isolated from aspirated fat preserved at 4°C for 0, 1, 2, and 3 days and flow cytometric analyses were performed on adipose-derived stem cells after culture for 1 week. Few differences in expression profile of principal surface markers were observed among groups, suggesting that biological properties of adipose-derived stem cells do not change by preservation at 4°C for up to 3 days. Vales are mean ± SE.

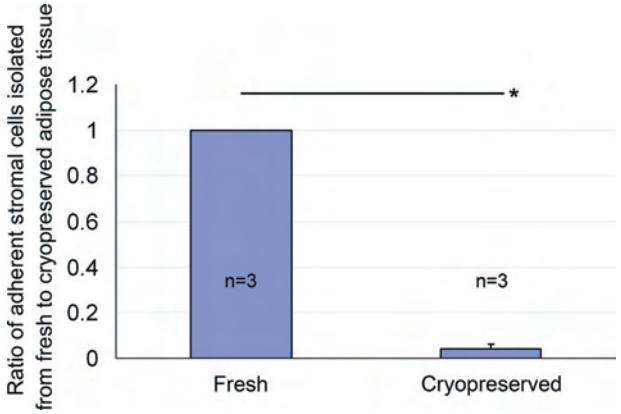

Fig. 6. Adipose-derived stem cell yields from cryopreserved liposaspirates. Fresh aspirated adipose tissue was mixed with an equal amount of freezing medium, cooled to −80°C in a programmable freezing system, and stored at −80°C for 1 month. The cryopreserved adipose tissue was thawed and processed to isolate adipose-derived stem cells, which were then cultured for 1 week. The ratio of adipose-derived stem cell yield to control (fresh liposaspirates) was calculated. Data were obtained from three patients, and statistical analysis was performed using paired *t* tests between groups. Adipose-derived stem cells were isolated from cryopreserved aspirated fat ($6.7 \pm 4.7 \times 10^4$ cells/ml after 1 week of culture), but the yield was much less than that of the fresh fat. Values are mean ± SE; $*p < 0.05$.

release of leaked intracellular oil from damaged adipocytes; the oil might be trapped in the surrounding matrices, proving difficult to measure using the oil ratio assay. Thus, the present result indicated that preservation at room temperature for 4 hours significantly damaged adipocytes in aspirated fat; thus, lipotransfer should be performed as quickly as possible after aspiration, especially when a large volume of aspirated fat is to be transplanted.

Since the reports showing that adipose tissue contains multipotent stem cells,[4,5] aspirated adipose tissue has been regarded as not only a filler material but also as an abundant source of stem cells. Adipose-derived stem cells reside in adipose tissue as progenitors of adipocytes, but it has been suggested that adipose-derived stem cells can differentiate into vascular endothelial cells,[13,14] can release angiogenetic factors under hypoxic conditions,[15] and can contribute to a higher graft take of transplanted fat.[14,16] In the current study, adipose-derived stem cell yield was maintained up to 4 hours at room temperature, and an adipose-derived stem cell yield similar to that of fresh aspirated fat was obtained from that preserved at 4°C for 24 hours. This finding indicates that a 1-day delay in isolation of adipose-derived stem cells from aspirated fat can be appropriate when the tissue is stored in a refrigerator. Therefore, overnight cooling transportation of aspirated fat to a specialized cell-processing center for isolation and banking of adipose-derived stem cells can be regarded as practical, although adipose-derived stem cell yield after 2 or 3 days would be uncertain, even with preservation at 4°C.

Isolated adipose-derived stem cells can be frozen, thawed, and cultured again as well as almost any other cell type. However, whether aspirated fat tissue can be frozen as an effective filler material or a source of adipose-derived stem cells has not yet been established. In this study, we tried to isolate adipose-derived stem cells from aspirated fat cryopreserved for 30 days and from fresh aspirated fat. The adipose-derived stem cell yield from cryopreserved fat was much lower than that of fresh aspirated fat. We tried several kinds of freezing conditions (rapid or slow freezing) and other freezing media (Dulbecco's Modified Eagle Medium or M-199 containing 10% dimethyl sulfoxide with 1% methylcellulose or 1% trehalose or 1 to 20% gelatin, or their mixture), but the adipose-derived stem cell yield was not improved (data not shown). Although there were a number of red blood cells contaminating cell fractions isolated from fresh aspirated fat, almost no red blood cells contaminated those from cryopreserved aspirated fat. Our result with adipose-derived stem cell yield from cryopreserved fat contradicts a recent report[17] showing that the adipose-derived stem cell yield from cryopreserved lipoaspirates was approximately 90 percent of that from fresh lipoaspirates. The reported adipose-derived stem cell yield from cryopreserved adipose is $3.7 \pm 1.4 \times 10^5$ cells/ml after 2 weeks of culture,[17] which is comparable to our result ($6.7 \pm 4.7 \times 10^4$ cells/ml after 1 week of culture), because adipose-derived stem cells proliferate 10 to 100 times in a week, depending on culture conditions. However, the reported adipose-derived stem cell yield from fresh adipose ($4.1 \pm 1.4 \times 10^5$ cells/ml after 2 weeks of culture[17]) was much less than that in this study ($7.9 \pm 1.5 \times 10^5$ cells/ml after 1 week of culture). It is unknown why the reported adipose-derived stem cell yield from fresh adipose differs between the two studies, but it may be the result of different methods of cell isolation.

CONCLUSIONS

We have demonstrated how the adipose-derived stem cell yield from aspirated fat changes depending on preservation conditions and time periods. Preservation for 4 hours at room temperature significantly damaged adipocytes but did not significantly alter adipose-derived stem cell yield. Adipose-derived stem cell yield significantly decreased with preservation for 24 hours at room temperature but not with preservation at 4°C. Thus, aspirated fat can be transported to a cell-processing center for cell isolation on the day after harvesting and for subsequent banking if it is kept at 4°C. Adipose-derived stem cell yield from cryopreserved aspirated fat was minimal, and a further optimization of methodology of freezing and preservation is needed for practical use of cryopreservation of aspirated fat intended as an adipose-derived stem cell source.

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DISCLOSURE

None of the authors has any commercial associations or financial interests that might pose or create a conflict of interest with information presented in this article.

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