Biological Properties and Therapeutic Value of Cryopreserved Fat Tissue

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Background: Fat grafting frequently requires multiple treatments and thus repeated liposuction to achieve treatment goals. The purpose of this study was to evaluate whether cryopreservation of adipose tissue may facilitate future fat grafting.
Methods: Lipoaspirates were harvested from six women and preserved using two cryopreservation methods: (1) simple cooling to −80°C (cryo-1); or (2) programmed cooling to −196°C (cryo-2). Fresh fat, cryo-1 fat, and cryo-2 fat were analyzed both in vitro and in vivo.
Results: Immunohistochemistry of both types of cryopreserved adipose tissue revealed that most adipocytes were necrotic. The cell number and viability of stromal vascular fraction cells were significantly decreased in cryo-1 fat (1.7×10^5 cells, 42.6 percent viable) and cryo-2 fat (2.0×10^5 cells, 55.4 percent viable), compared with fresh fat (3.9×10^5 cells, 90.6 percent viable). Although adipose-derived stem cells were cultured successfully from all fats, functional adipose-derived stem cells from cryopreserved fats were much fewer, with comparable multilineage differentiating capacity. In vivo studies using human fat grafted into immunocompromised mice revealed that, 3 months after transplantation, all of the cryopreserved fats maintained their volume to some extent; however, the cryopreserved fats were mostly filled with dead tissue and produced significantly lower engraftment scores than fresh fat.
Conclusions: Most adipocytes were killed in the process of cryopreservation and thawing. Adipose-derived stem cells were isolated from cryopreserved fat, but the number of functional adipose-derived stem cells was very limited in both cryopreservation methods. After grafting, cryopreserved fat was retained as dead and fibrous tissue, suggesting a risk of clinical complications such as oil cysts. (*Plast. Reconstr. Surg.* 141: 104, 2018.)

Fat grafting for reconstruction, revitalization, or aesthetics usually requires multiple treatments to achieve treatment goals. Because harvested adipose tissue is not vascularized and becomes necrotic within hours,[1] autologous fat grafting must be performed immediately after liposuction.[2] Although some clinicians cryopreserve excess harvested fat for subsequent use,[3–5] this technique remains controversial because of insufficient evidence supporting its use. If necrotic fat is grafted, the result is not only a high rate of graft absorption but also serious complications such as oil cyst formation, chronic inflammation, and progressive calcification.[6]

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There have been many studies on clinical and experimental cryopreservation of fat tissue showing controversial results.[3–5,7–13] The controversy appears to result from the difficulty of correctly evaluating adipocyte viability in cryopreserved and grafted fat tissues. In this regard, we have used an established, reliable method for adipocyte viability—an immunostaining agent

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against a cytoplasm marker for viable adipocytes, perilipin.[14,15] By using this method, we recently succeeded in showing convincing evidence for adipocyte turnover and tissue regeneration after fat grafting.[16,17] Here, we investigated whether either of two cryopreservation protocols for adipose tissue could be a reliable technique for future clinical use.

MATERIALS AND METHODS

Preparation of Cryopreserved Fat

Human lipoaspirates were obtained from six healthy female donors aged 41.4 ± 5.3 years (mean ± SD) with an average body mass index of 21.5 ± 1.8 kg/m^2 who underwent conventional liposuction of the abdomen or thigh. Each patient provided written informed consent, and the research protocol was approved by the institutional review board.

A portion of the aspirated fat was centrifuged (1200 *g* for 3 minutes) to discard the oil and water layers, and the resulting fat layer was termed "fresh fat." The remainder of the aspirated fat tissue was cryogenically stored using one of two methods. For the first method (cryo-1 fat), aspirated fat within a disposable syringe was simply put in a deep freezer set to −80°C without any cryoprotectant. For the second method (cryo-2 fat), aspirated fat was cryopreserved in a controlled rate freezer (Kryo-560; Planer Plc, Middlesex, United Kingdom). The fat tissue placed in an adipose tissue cryopreservation medium (ACSelerate CP; American Cryostem, Eatontown, N.J.) was subjected to programed freezing with 1°C/minute freezing steps until −80°C and stored in liquid nitrogen at −196°C. After 2 weeks, the stored fat was thawed in 37°C water and centrifuged (1200 *g* for 3 minutes). The volume and weight of each product was measured, and the specific gravity was calculated. Figure 1 shows a schematic of the two cryopreservation methods.

Whole-Mount Staining of Living Fat Tissue

Fat samples were cut into 3-mm pieces and incubated with BODIPY 558/568 (Molecular Probes, Eugene, Ore.) and Hoechst 33342 (Dojindo, Kumamoto, Japan) for 30 minutes to stain adipocytes and nuclei, respectively. In addition, Alexa Fluor 488–conjugated isolectin GS-IB4 (Molecular Probes) or propidium iodide (Sigma-Aldrich, St. Louis, Mo.) was used to stain vascular endothelial cells or nuclei of necrotic cells, respectively. The samples were then washed and imaged with a confocal microscope (Leica TCS SP2; Leica Microsystems GmbH, Wetzlar, Germany). Fifty images acquired at 0.4-μm intervals were used to reconstruct 20 μm-thick three-dimensional images.

Immunohistochemistry

To evaluate adipocyte viability, fat samples were fixed (Zinc Fixative; BD Biosciences, San Jose, Calif.), paraffin-embedded, sectioned at 5 μm, and immunostained with primary antibody guinea pig anti-perilipin (dilution, 1:200; Progen, Heidelberg, Germany), which stains only viable adipocytes, and secondary antibody Alexa Fluor 647–conjugated goat anti–guinea pig immunoglobulin G (dilution, 1:200; Invitrogen, Carlsbad, Calif.). Nuclei and endothelial cells were stained with Hoechst 33342 (dilution, 1:200; Dojindo) and Alexa Fluor 488–conjugated lectin (dilution, 1:200; Invitrogen), respectively.

A part of each fat was organ-cultured at 37°C in 5% carbon dioxide for 24 hours with serum-free Dulbecco's Modified Eagle Medium with Ham's F-12 (Wako Pure Chemical, Osaka, Japan) and subsequently fixed and immunostained in the same manner as above. Stained sections were examined with a fluorescence microscope (Keyence, Osaka, Japan).

Stromal Vascular Fraction Isolation and Flow Cytometry

The stromal vascular fraction was isolated from the fat samples as described previously.[18] Briefly, each tissue was washed and digested in phosphate-buffered saline containing 0.075% collagenase (Wako Pure Chemicals) for 30 minutes at 37°C in a shaking water bath. After centrifugation (800 *g* for 10 minutes) and resuspension of the cell pellets, the stromal vascular fraction was obtained by filtering the cell suspension through a series of 100-, 70-, and 40-μm meshes (Millipore, Billerica, Mass.). Cell numbers and viability were measured with an automated cell counter (NucleoCounter NC-100; ChemoMetec, Allerød, Denmark).

The stromal vascular fraction samples were examined by flow cytometry using the following monoclonal antibodies conjugated to fluorochromes: anti–CD31-phycoerythrin, anti–CD34-phycoerythrin-Cy7, and anti–CD45-fluorescein isothiocyanate (BD Biosciences). Cells were incubated with monoclonal antibodies (dilution, 1:10) for 30 minutes. Multicolor flow cytometry was performed with an MACS-Quant (Miltenyi Biotec, Bergisch Gladbach, Germany), and cell composition was calculated.

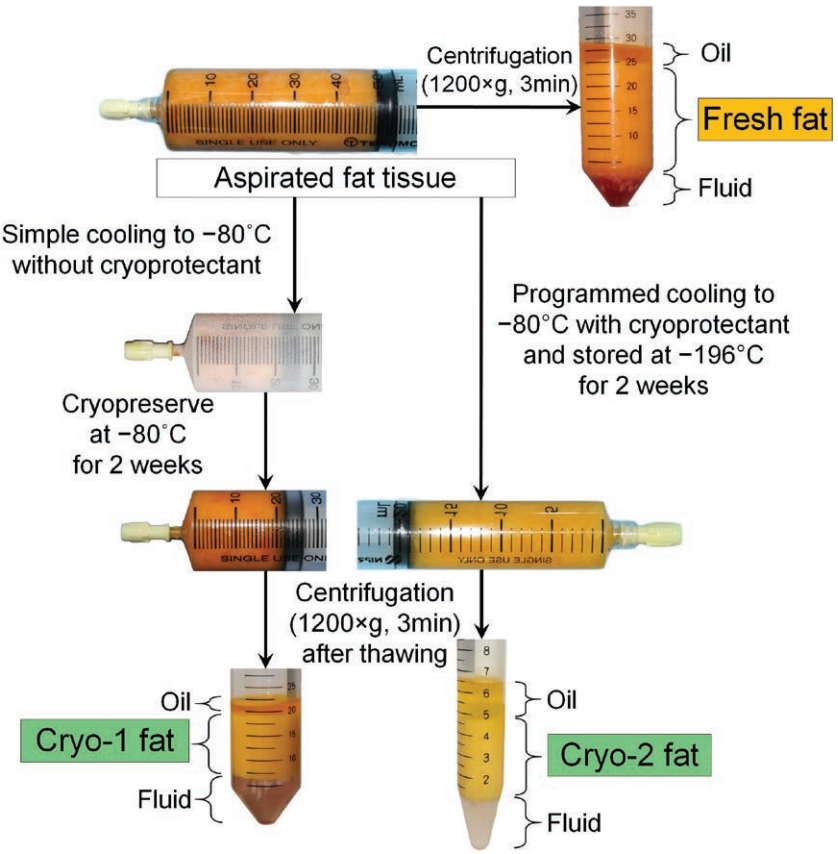

Fig. 1. Schematic representation of the cryopreservation methods. Aspirated fat was centrifuged, and the resulting fat layer was termed fresh fat. Alternatively, aspirated fat was frozen and stored in two ways: (1) directly set in a deep freezer set at −80°C without any cryoprotectant; or (2) cooled with a programmed protocol (−1°C/minute) and stored in liquid nitrogen at −196°C with cryoprotective agents. After 2 weeks, the fat was thawed in 37°C water and centrifuged. The former was termed "cryo-1 fat" and the latter "cryo-2 fat." After thawing in warm water, cryo-1 and cryo-2 fats were centrifuged and the fatty layer was used for further experiments. All centrifugation was performed at 1200 *g* for 3 minutes.

Stromal Vascular Fraction Cell Culture

The isolated stromal vascular fraction from each fat sample was cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 supplemented with 10% fetal bovine serum, 100 IU penicillin, and 100 mg/ml streptomycin at 37°C in 5% carbon dioxide and humidified air. The medium was replaced every 3 days, and after 14 days in primary culture, the attached cells were trypsinized, counted, and defined as adipose-derived stem cells at passage 0.

Colony-Forming Assay

Passage 0 cultured adipose-derived stem cells were seeded at initial seeding numbers of 3×10, 1×10^2, or 3×10^2 cells. Cultures were incubated at 37°C for 7 days, then fixed with methanol, stained with a 5% Giemsa solution, and counted using digital imaging software (Photoshop; Adobe Systems, Inc., San Jose, Calif.). A colony-forming score was calculated (i.e., colony-forming score = number of colonies/number of seeded cells × yielded cultured cells from 1 g of adipose tissue).

Multilineage Differentiation Assay

Passage 0 adipose-derived stem cells were first incubated in Dulbecco's Modified Eagle Medium/Ham's F-12 until confluent. Adipogenic and osteogenic differentiation were initiated using adipogenic medium (Adipolife; Lifeline Cell Technology, Frederick, Md.) or osteogenic medium (Osteolife; Lifeline Cell Technology). After culturing for 21 days with medium changes every 3 days, differentiation ability was analyzed qualitatively with Oil Red staining and quantitatively with AdipoRed reagent (Lonza, Basel,

Switzerland) for adipogenesis, or qualitatively by means of von Kossa staining and quantitatively by using the Calcium-E test (Wako Pure Chemicals) for osteogenesis. Chondrogenic differentiation was conducted using a micromass culture system: adipose-derived stem cells were resuspended in chondrogenic medium (Chondrolife; Lifeline Cell Technology) and centrifuged (800 *g* for 5 minutes). Half of the medium was changed after centrifugation every 2 days. After 2 weeks, chondrogenic ability was analyzed qualitatively by means of Alcian blue staining and quantitatively by measurement of micromass diameter.

Fat Grafting into Mouse Models

All animal experiments were performed in accordance with Use Committee of the University of Tokyo animal guidelines. Eight-week-old BALB/c mice were obtained from Japan CLEA, Inc. (Tokyo, Japan). After anesthetization with 2.5% isoflurane, fresh, cryo-1, or cryo-2 fat samples were injected linearly with an injection volume of approximately 1 ml/cm beneath the back skin (mobile area) and the scalp (nonmobile area) of 18 mice ($n = 6$ for each fat type) through an 18-gauge blunt cannula. Three months postoperatively, the grafted fat samples were harvested and weighed to calculate the normalized sample weight (harvested sample weight/total body weight). Samples were fixed (Zinc Fixative) and paraffin-embedded for immunohistochemistry. All animals were cared for in accordance with the institutional guidelines. Viable adipocyte area was measured by evaluating the perilipin-stained area in sections. An engraftment score was calculated (i.e., normalized fat sample weight/normalized initial sample weight × percentage of viable adipocyte area) to evaluate the magnitude of true graft retention.

Statistical Analysis

Data were analyzed using IBM SPSS Version 23.0 (IBM Corp., Armonk, N.Y.) and Kyplot 2.0 (freeware). Based on the Kolmogorov-Smirnov test, our data were distributed normally within the donor population. Therefore, one-way analysis of variance with Bonferroni adjustment for post hoc comparisons was used to determine significant differences between the means ($p < 0.05$ unless otherwise stated).

RESULTS

Visualization and Viability Assessment of Cryopreserved Fat

Visualization by whole-mount staining revealed no major differences in three-dimensional structures among fresh fat, cryo-1 fat, and cryo-2 fat (Fig. 2, *above*). Double staining with Hoechst/propidium iodide revealed that, although most nuclei in fresh fat were alive (Hoechst-positive and propidium iodide–negative), both cryopreserved fats contained many dead nuclei (Hoechst/propidium iodide–double positive), indicating severe tissue damage caused by cryopreservation and thawing (Fig. 2, *below*).

To evaluate adipocyte viability, we performed immunostaining for fresh fat just after harvesting, and cryo-1 fat and cryo-2 fat just after thawing. Although fresh and cryo-1 fats had round, perilipin-positive cells, suggesting adipocyte viability, no such perilipin-positive cells were detected in cryo-2 fat. [**See Figure, Supplemental Digital Content 1**, which shows immunohistochemistry of adipose tissue before transplantation. Immediately after harvesting or thawing (*above*), round cells that were strongly positive for perilipin were seen in fresh and cryo-1 fat, but not in cryo-2 fat. When the samples were fixed after 24 hours of organ culture (*center*), fresh fat retained cells expressing perilipin, whereas cryo-1 fat lacked perilipin expression, suggesting that only fresh fat contains viable adipocytes. *Scale bars* = 100 μm. (*Below*) Percentage of viable adipocyte area after 24 hours of organ culture. Perilipin-positive adipocytes were judged as viable ones. Bonferroni-corrected $p < 0.0001$ (fresh versus cryo-1, fresh versus cryo-2) and $p = 1$ (cryo-1 versus cryo-2). *Bonferroni-corrected $p < 0.05$. *NS*, not significant, ***http://links.lww.com/PRS/C490***.] Next, to determine whether perilipin-positivity in cryo-1 fat was truly indicative of adipocyte survival, each fat was immunostained after 24 hours of organ culture. Unlike fresh fat, cryo-1 fat lost its perilipin expression, indicating that, unlike programmed cooling, rapid cooling maintained perilipin protein structures but adipocytes were already killed by the freezing (**see Figure, Supplemental Digital Content 1**, *center* and *below*, ***http://links.lww.com/PRS/C490***). Therefore, it was found that cryopreservation kills the most adipocytes regardless of cooling speed.

Characteristics of Cryopreserved Adipose Tissue and Flow Cytometry

We detected no significant differences in the specific gravity among fresh fat (0.95 ± 0.03; mean ± SD), cryo-1 fat (0.97 ± 0.03), and cryo-2 fat (0.96 ± 0.02). The total cell number and viability of stromal vascular fraction cells from 1 ml of fat tissue were significantly decreased in cryo-1 fat [$1.7 \pm 0.5 \times 10^5$ cells ($p < 0.0001$), 42.6 ± 13.2 percent viable ($p < 0.0001$)], and cryo-2 fat [$2.0 \pm 0.6 \times 10^5$ cells ($p = 0.00031$),

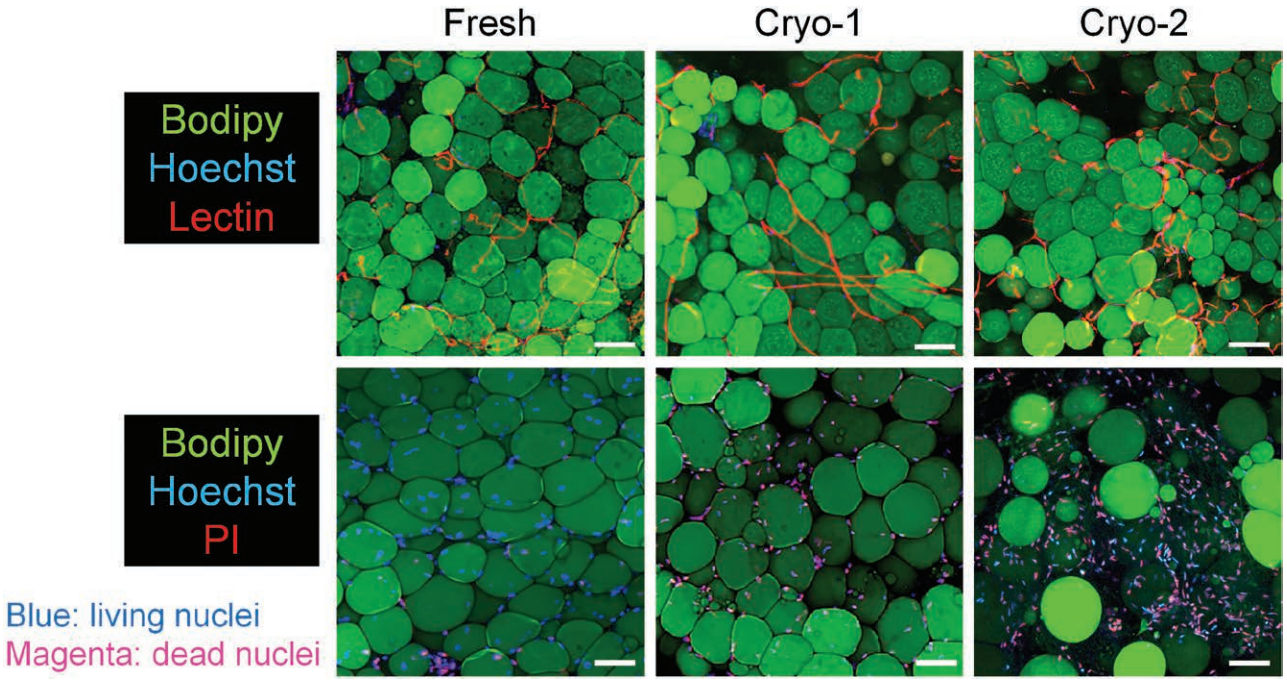

Fig. 2. Whole mount staining of raw adipose tissue with BODIPY, lectin, and Hoechst was performed to visualize lipid, capillaries, and nuclei, respectively. Three-dimensional images revealed no major differences in three-dimensional structures among fresh, cryo-1, and cryo-2 fats. In Hoechst/propidium iodide (*PI*) double staining, although over 90 percent of nuclei in fresh fat were alive, cryo-1 and cryo-2 fats contained a huge number of dead nuclei, suggesting severe tissue damage by cryopreservation. *Scale bars* = 100 μm.

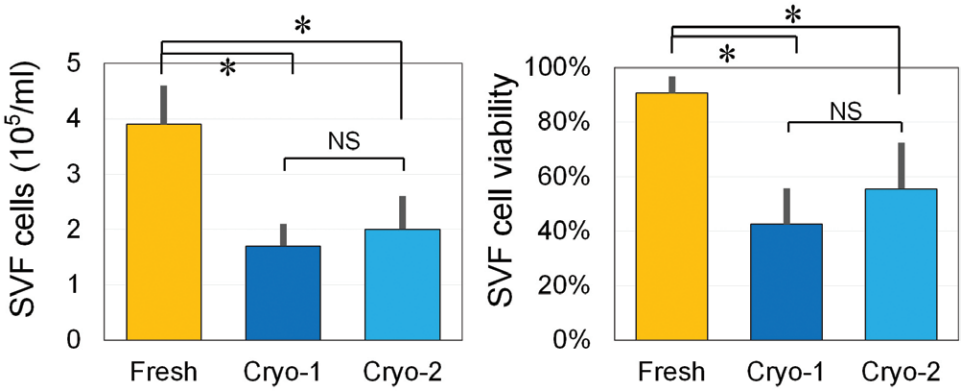

Fig. 3. Cellular analyses of stromal vascular fraction (*SVF*). The total number and viability of the stromal vascular fraction cells obtained from 1 ml of fat were significantly lower in cryo-1 and cryo-2 fats compared with fresh fat. (*Left*) Bonferroni-corrected $p < 0.0001$ (fresh versus cryo-1), $p = 0.00031$ (fresh versus cryo-2), and $p = 0.94$ (cryo-1 versus cryo-2). (*Right*) Bonferroni-corrected $p < 0.0001$ (fresh versus cryo-1), $p = 0.00066$ (fresh versus cryo-2), and $p = 0.30$ (cryo-1 versus cryo-2). *Bonferroni-corrected $p < 0.05$. *NS*, not significant.

55.4 ± 17.1 percent viable ($p = 0.00066$)], compared with fresh fat ($3.9 \pm 0.7 \times 10^5$ cells, 90.6 ± 3.3 percent viable) (Fig. 3). The reduction in the total cell number in cryopreserved fats indicates that some cells died and enucleated before being detected by the automated cell counter.

Flow cytometric analysis of stromal vascular fraction showed that the number of $CD45^+$ hematopoietic cells was decreased in cryo-1 ($1.7 \pm 0.7 \times 10^5$) and cryo-2 fat ($3.4 \pm 1.3 \times 10^5$) compared with fresh fat ($11.5 \pm 2.9 \times 10^5$). [**See Figure, Supplemental Digital Content 2**, *above*, which shows flow cytometric analysis of stromal vascular fraction. (*Above* and *center*) Representative plots of flow cytometric data. $CD45^+$ hematopoietic cells (*red*) were greatly decreased in cryo-1 and cryo-2 fats compared with fresh fat. (*Below*) Cell number of each cellular component in the stromal vascular fraction. Not all

cells are considered alive/functional, especially in cryo-1 and cryo-2 fats. Adipose-derived stem cells (*ASCs*) are $CD45^-/CD31^-/CD45^+$ and vascular endothelial cells (*ECs*) are $CD45^-/CD31^+/CD34^+$. *HC*, hematopoietic cells, ***http://links.lww.com/PRS/C491***.] The $CD45^-$ component was further separated into $CD45^-/CD31^+/CD34^-$ endothelial cells, $CD45^-/CD31^+/CD34^+$ adipose-derived stem cells, and $CD45^-/CD31^-/CD34^-$ other cells: the cellular distribution among these cell populations did not remarkably change among the three fats, except that cryo-2 fat contained a slightly larger proportion of endothelial cells and other cells (**see Figure, Supplemental Digital Content 2**, *center* and *below*, ***http://links.lww.com/PRS/C491***). The number of adipose-derived stem cells in cryo-1 ($8.5 \pm 2.1 \times 10^5$) and cryo-2 fat ($7.8 \pm 3.2 \times 10^5$) was more than half that of fresh fat ($13.4 \pm 2.5 \times 10^5$); however, many were found dead or nonfunctioning by the following experiments.

Stromal Vascular Fraction Cell Culture and Function Analysis

We generated adherent cultures of stromal vascular fractions to measure viable adipose-derived stem cells contained in each fat. The number of adherent cells cultured from the stromal vascular fraction of cryo-1 and cryo-2 fats were markedly decreased compared with that of fresh fat. (**See Figure, Supplemental Digital Content 3**, which shows the primary culture of stromal vascular fraction. Microscopic views of the cell culture from each fat type at the indicated days after seeding an equal number of stromal vascular fraction cells. Adipose-derived stem cells were selectively expanded in the culture dish. *Scale bars* = 100 μm, ***http://links.lww.com/PRS/C492***.) Although some of the cells from the stromal vascular fraction of cryopreserved fats showed normal adipose-derived stem cell–like morphology, others exhibited a feathering-like shape and stopped growing (Fig. 4, *above*). These adipose-derived stem cells were considered barely alive and thus nonfunctional. After 2 weeks, the number of adipose-derived stem cells was significantly greater in cultures derived from fresh fat ($7.5 \pm 1.6 \times 10^6$) than from cryo-1 [$1.5 \pm 0.6 \times 10^6$ ($p = 0.00021$)] or cryo-2 fat [$1.3 \pm 0.6 \times 10^6$ ($p = 0.00017$)] (Fig. 4, *below, left*). Next, we calculated the number of adipose-derived stem cells obtained from a 2-week culture "from 1 g of fat" derived from fresh fat ($6.2 \pm 0.2 \times 10^6$ cells), cryo-1 fat ($0.5 \pm 0.1 \times 10^6$ cells), or cryo-2 fat ($0.5 \pm 0.1 \times 10^6$ cells). Thus, cryopreserved adipose tissue contains viable adipose-derived stem cells but far fewer than fresh fat.

Colony-Forming Assay

At the initial concentration of 3×10^2 cells per well, we detected significantly higher number of colonies in fresh fat than in cryo-1 and cryo-2 fat. (**See Figure, Supplemental Digital Content 4**, which shows the colony-forming assay. (*Left*) Adipose-derived stem cells obtained from each fat type were seeded at three initial seeding numbers. The number of colonies after 7 days in culture were plotted with linear approximation ($n = 6$). At the initial concentration of 3 × 102 cells per well, a significant difference in the number of colonies was detected between fresh fat and cryo-1 fat, but not between cryo-1 fat and cryo-2 fat. (*Right*) Macroscopic colonies were fixed with methanol and stained with 5% Giemsa solution after 7 days in culture. *Scale bars* = 1 cm, ***http://links.lww.com/PRS/C493***.) We calculated a colony-forming score to estimate the number of functional adipose-derived stem cells yielded by fresh fat (1.9×10^8), cryo-1 fat (2.6×10^7), and cryo-2 fat (1.5×10^7) (Fig. 4, *below, right*).

Multilineage Differentiation Assay

After inducing differentiation, adipose-derived stem cells from all three fat types displayed similar adipogenic, osteogenic, and chondrogenic differentiation capabilities, as confirmed by Nile Red, von Kossa, and Alcian blue staining, respectively. [**See Figure, Supplemental Digital Content 5**, which shows the multilineage differentiation assay. (*Above*) Microscopic images of differentiated cells derived from cultured human adipose-derived stem cells of fresh fat, cryo-1 fat, and cryo-2 fat show that the three fat types yielded similar adipogenic, osteogenic, and chondrogenic differentiation. Lineage-specific differentiation is delineated by Nile Red (adipogenic), von Kossa (osteogenic), and Alcian blue (chondrogenic) stains. *Scale bars* = 100 μm. (*Below*) Plots showing quantitative analyses indicate that the capacity for multilineage differentiation, intracellular lipid content (adipogenesis), and calcium deposition (osteogenesis) were significantly higher in fresh fat than in cryo-1 fat and cryo-2 fat, whereas no significant difference was found in micromass diameter (chondrogenesis) between the three fats. (*Below, left*) Relative fluorescent units (adipogenesis); Bonferroni-corrected $p < 0.0001$ (fresh versus cryo-1, fresh versus cryo-2), and $p = 1$ (cryo-1 versus cryo-2). (*Below, center*) Calcium (osteogenesis); Bonferroni-corrected $p = 0.013$ (fresh versus cryo-1), $p = 0.010$ (fresh versus cryo-2), and $p = 0.85$ (cryo-1 versus cryo-2). (*Below, right*) Cartilage diameter (chondrogenesis); Bonferroni-corrected $p = 0.099$ (fresh

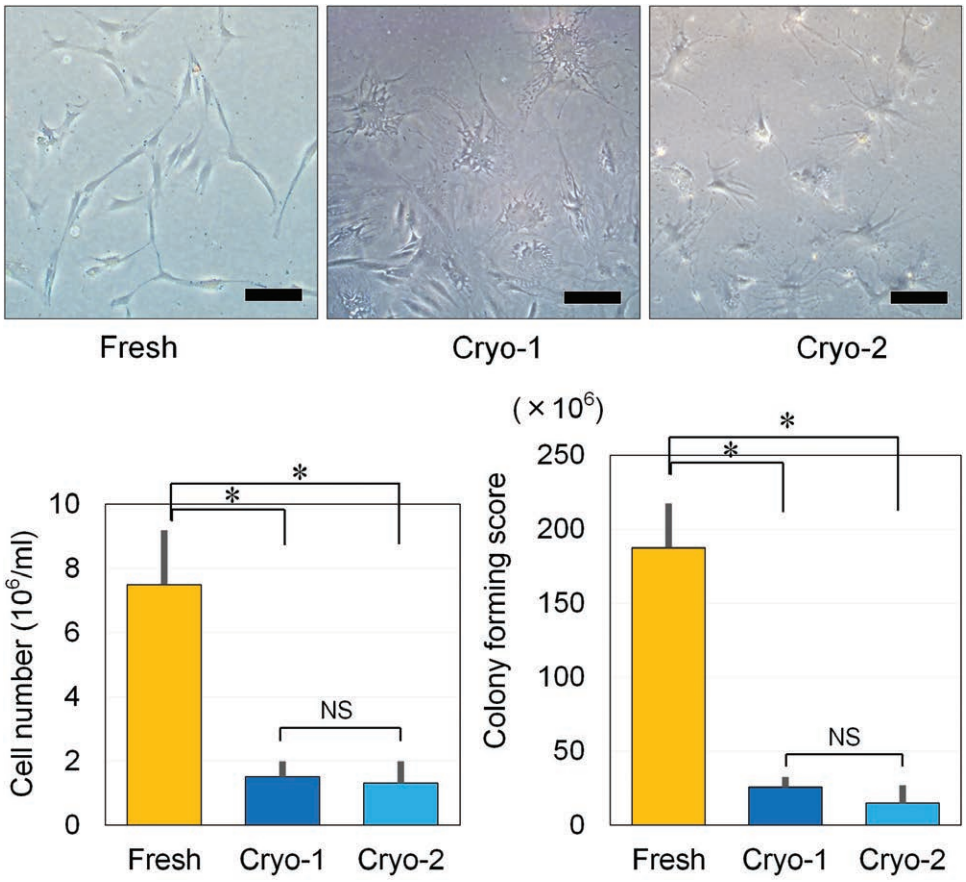

Fig. 4. Primary culture of stromal vascular fraction and colony-forming assay. (*Above*) Cell yields obtained after 2 weeks of culture. Bonferroni-corrected $p = 0.00021$ (fresh versus cryo-1), $p = 0.00017$ (fresh versus cryo-2), and $p = 0.98$ (cryo-1 versus cryo-2). *Bonferroni-corrected $p < 0.01$. *NS*, not significant. (*Below*, *left*) Microscopic views of selected samples on day 9. Adherent cells isolated from cryopreserved fats occasionally showed abnormal (feathering-like) shape and did not grow any further. *Scale bars* = 100 μm. (*Below, right*) The colony-forming score (i.e., number of colonies/number of seeded cells × yielded cultured cells from 1 g of adipose tissue) was dramatically higher in fresh fat than in cryo-1 fat and cryo-2 fat. *Bonferroni-corrected $p < 0.05$. *NS*, not significant. Bonferroni-corrected $p < 0.0001$ (fresh versus cryo-1, fresh versus cryo-2) and $p = 0.90$ (cryo-1 versus cryo-2). *Bonferroni-corrected $p < 0.01$. *NS*, not significant.

versus cryo-1), $p = 0.051$ (fresh versus cryo-2), and $p = 0.92$ (cryo-1 versus cryo-2), ***http://links.lww.com/PRS/C494***.] In quantitative analyses, fresh fat cells had a significantly higher intracellular lipid content [reflecting adipogenesis, $p < 0.0001$ (fresh versus cryo-1 and cryo-2)] and calcium deposition [osteogenesis, $p = 0.023$ (fresh versus cryo-1) and $p = 0.010$ (fresh versus cryo-2)] than cryo-1 and cryo-2 fat, whereas no significant difference was found in micromass diameter (chondrogenesis) among the three fat types [$p = 0.099$ (fresh versus cryo-1), $p = 0.051$ (fresh versus cryo-2), and $p = 0.92$ (cryo-1 versus cryo-2)] (**see Figure, Supplemental Digital Content 5**, *below*, ***http://links.lww.com/PRS/C494***). Overall, adipose-derived stem cells cultured from stromal vascular fraction of cryopreserved fat showed a reduced but still valid capacity for multilineage differentiation.

Fat Grafting into Mice Models

Each fat type was injected into immunocompromised mice (Figs. 5 and 6). The injection volumes beneath the back skin (mobile area) and scalp (nonmobile area) were 0.37 ± 0.04 ml and 0.10 ± 0.02 ml, respectively (mean ± SD). After 3 months, each fat was surgically harvested. The cryopreserved fat, especially cryo-2 fat, was visibly smaller and contained more oil than fresh fat (Fig. 5, *right*). Normalized sample weights, which reflect volume retention, were significantly different between cryo-2 fat and the other two fats injected in the back [$p < 0.0001$ (versus fresh),

$p = 0.00015$ (versus cryo-1)], and between cryo-2 fat and fresh fat in the scalp ($p = 0.013$) (Fig. 6).

To assess adipocyte viability, we performed immunostaining for adipose tissue 3 months after grafting. As shown by the representative images (Fig. 7), adipocytes of fresh fat maintained their original size, shape, and strong expression of perilipin, although a few oil droplets surrounded by macrophages lacking perilipin staining (so-called crown-like structures[19]) were also seen. (**See Figure, Supplemental Digital Content 6**, which shows the high-magnification images of fat graft sections shown in Figure 7. *White scale bars* = 100 μm. *vWF*, von Willebrand factor, ***http://links.lww.com/PRS/C495***.) Cryo-1 and cryo-2 fats formed large oil drops (generated from multiple necrotic adipocytes) and exhibited considerable fibrosis. At the same time, new small (<20 μm) perilipin-positive preadipocytes appeared in the cryopreserved fats, suggesting adipocyte regeneration that is incomplete and at a very small magnitude.

To evaluate the true retention of fat grafts, we calculated "engraftment scores" (Fig. 6). The perilipin-positive adipocyte-surviving zone was considerably larger in fresh fat (87.8 ± 7.9 percent) than in cryo-1 (5.3 ± 2.3 percent) and cryo-2 fat (8.8 ± 2.9 percent). The engraftment score of fresh fat grafted into the back (43.8 ± 7.9 percent) was dramatically larger than those of cryo-1 (2.1 ± 0.9 percent) and cryo-2 fat (0.9 ± 0.5 percent) ($p < 0.0001$ for fresh versus cryo-1 and cryo-2). Also, the engraftment score of fresh fat grafted into the scalp (72.0 ± 7.1) was considerably larger than those of cryo-1 (3.5 ± 1.1) and cryo-2 fat (4.3 ± 1.4) ($p < 0.0001$ for fresh versus cryo-1 and cryo-2).

DISCUSSION

Considering growing demand for fat grafting in recent years, cryogenic preservation of excess harvested fat, if possible, would give us a great advantage in avoiding the time, effort, cost, and complications related to repeated liposuction. Some clinicians and researchers have claimed that the volume retention of grafted cryopreserved fat is comparable to that of fresh fat,[3–5,7] whereas others have reported negative results such as low recovery of adipose-derived stem cells after cryopreservation[8] and necrosis of cryopreserved fat grafted into animal models,[9] and many clinicians remain against the clinical use of frozen fat. Accordingly, we have conducted detailed analyses of cryopreserved fat to assess its biological viability and function and its therapeutic value.

Cryopreservation methods of "cells isolated from tissue" are established, and have been practiced extensively over many years. For example, stromal vascular fractions cryopreserved for 12 years have been shown to preserve cellular composition and adipose-derived stem cell function.[20] However, if a "tissue" is cryopreserved, freezing injury (mechanical damage from the intracellular/extracellular ice crystals and the increase in concentration of solutes[21]) occurs because the cryoprotectant cannot diffuse into each cell.[22] Therefore, in currently available cryogenic banking of various tissues such as skin,[23] heart valve,[24] and dura mater,[25] all cells in the tissue cannot be recovered after freezing and thawing, and the cryopreserved tissue serves only as tissue matrix (scaffold).[24,26]

There are many previous publications on the cryopreserved adipose tissue, but it seems that most of them have methodologic errors or

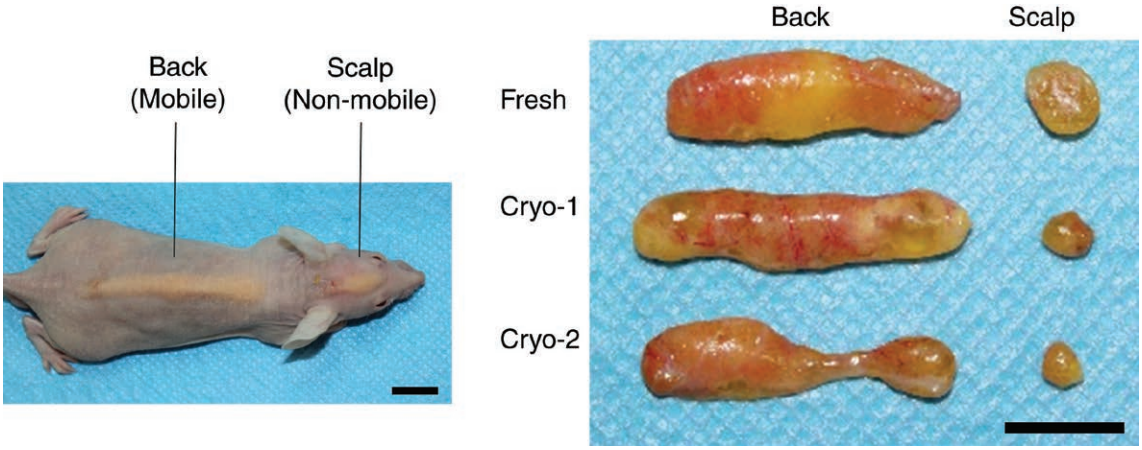

Fig. 5. Fat graft analysis using immunocompromised mice. (*Left*) Human fat (fresh, cryo-1, or cryo-2) was linearly injected into the indicated areas beneath the back skin and the scalp. *Scale bar* = 1 cm. (*Right*) Macroscopic views of harvested fat samples at 3 months after transplantation. *Scale bar* = 1 cm.

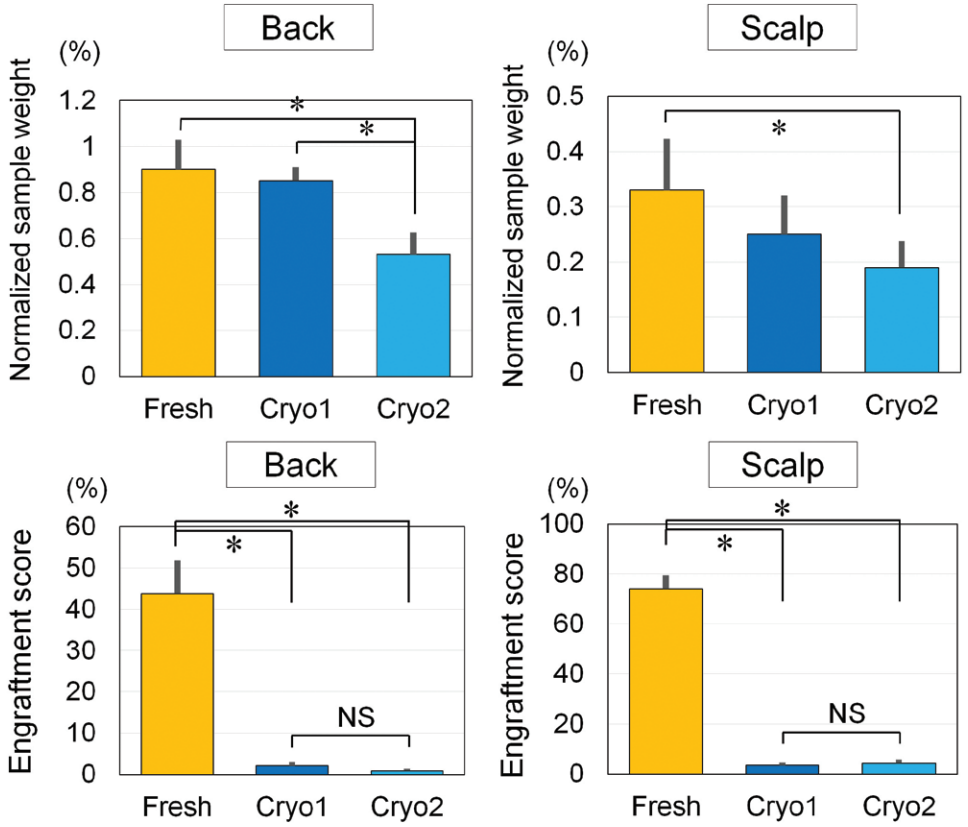

Fig. 6. Fat graft analysis using immunocompromised mice. (*Above*) Plots show retention of normalized sample weight (normalized final sample weight/normalized initial sample weight) of grafted fat samples. (*Above*, *left*) Bonferroni-corrected $p = 0.99$ (fresh versus cryo-1), $p < 0.0001$ (fresh versus cryo-2), and $p = 0.00015$ (cryo-1 versus cryo-2). (*Above*, *right*) Bonferroni-corrected $p = 0.23$ (fresh versus cryo-1), $p = 0.013$ (fresh versus cryo-2), and $p = 0.518$ (cryo-1 versus cryo-2). *Bonferroni-corrected $p < 0.05$. *NS*, not significant. (*Below*) Plots show engraftment scores (normalized sample weight × percentage of viable adipocyte area) that quantify the magnitude of true graft retention. Percentage of viable adipocyte area was measured using immunostained sections of each sample (see Fig. 7). (*Below*, *left*) Bonferroni-corrected $p < 0.0001$ (fresh versus cryo-1, fresh versus cryo-2) and $p = 1$ (cryo-1 versus cryo-2). (*Below*, *right*) Bonferroni-corrected $p < 0.0001$ (fresh versus cryo-1, fresh versus cryo-2) and $p = 1$ (cryo-1 versus cryo-2). *Bonferroni-corrected $p < 0.05$. *NS*, not significant.

limitations. For example, immunohistochemistry of "quickly frozen" adipose tissue "immediately after" thawing can falsely judge dead adipocytes as alive.[10] As we have shown in **Figure, Supplemental Digital Content 1**, ***http://links.lww.com/PRS/C490***, a certain amount of time (24 hours) is needed to dissolve cryopreserved proteins. Besides, volume retention or weight retention is not a reliable index with which to evaluate fat grafting results because oil cysts and fibrosis formed from fat necrosis also increase the volume and weight, as we show in Figure 7. Furthermore, routine histology of adipose tissue such as hematoxylin and eosin staining cannot distinguish dead adipocytes from living adipocytes, as we reported previously.[16] As a sensitive and reliable indicator of adipocyte viability, we strongly recommend immunostaining for perilipin, the most abundant protein on the surface of intracellular lipid droplets,[20] which easily identifies dead adipocytes where perilipin expression is lost.[6,27–32] There are other methods to measure viable cells, such as the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) carbonyl]-2*H*-tetrazoliumhydroxide (XTT) assay, the 3-(4,5-2-yl)-2,5-ditetrazolium bromide (MTT) assay,[33–35] and the glycerol-3-phosphate dehydrogenase assay.[36,37] Such enzymes can be measured even in dead cells if measurement is performed immediately after thawing of cells/tissues.

In whole-mount staining, dead nuclei in both cryopreserved fats indicated severe cell damage after cryopreservation. Cryo-2 fat lacked perilipin

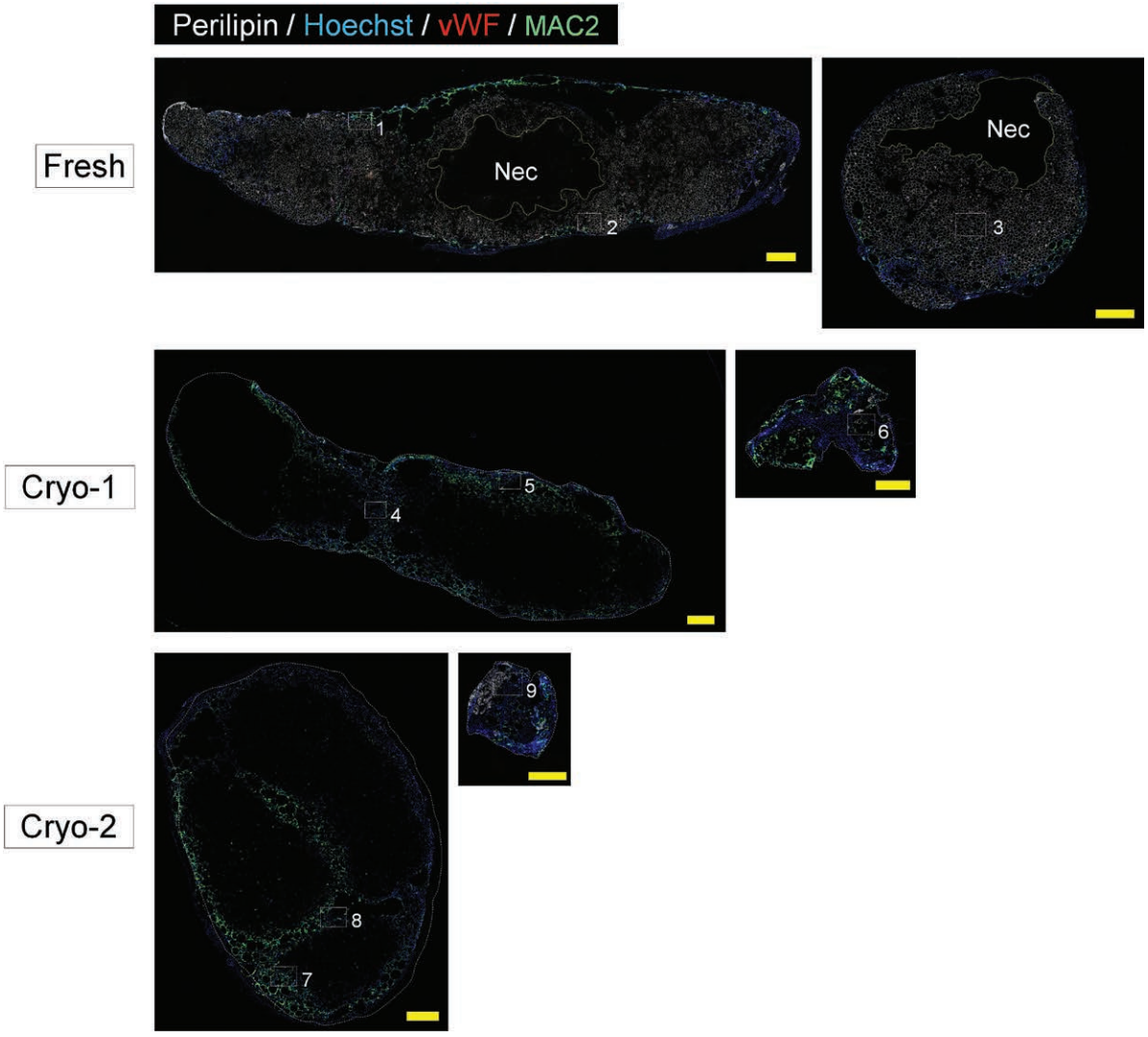

Fig. 7. Immunohistochemistry of grafted fat. Images show immunostained fat graft sections of fresh fat, cryo-1 fat, and cryo-2 fat 3 months after fat grafting (*white*, perilipin for viable adipocytes; *green*, MAC-2 for macrophages; *red,* von Willebrand factor for vascular endothelial cells; and *blue*, Hoechst 33342 for nuclei). (*Left*) Samples grafted in the back; (*right*) samples grafted under the scalp. *White dashed lines* outline cross-section edge of samples, and *yellow dashed lines* outline the central necrotic area (*Nec*) of grafted fresh fat. Highly magnified images of the *numbered white squares* are shown in **Supplemental Digital Content 6**, ***http://links.lww.com/PRS/C495***. *Yellow scale bars* = 1 mm. *vWF*, von Willebrand factor.

staining when fixed immediately after thawing. Although cryo-1 fat was perilipin-positive when fixed immediately after thawing, after 24 hours in culture, cryo-1 fat became perilipin-negative, suggesting that rapid cooling preserved perilipin protein structure temporarily, although adipocytes died in the freezing process. Therefore, we conclude that almost all adipocytes die during cryopreservation, regardless of cooling speed and the presence of cryopreservatives.

Next, we examined the viability and function of stromal vascular fraction cells obtained from cryopreserved fats. The number of living stromal vascular fraction cells was lower in cryopreserved fats than in fresh fat. Flow cytometry revealed a reduction in the number of blood-derived cells (leukocytes) from cryopreserved fats, suggesting a specific vulnerability of hematopoietic cells to freezing. We observed no significant difference in the cellular composition of stromal vascular fraction cells between cryo-1 and cryo-2 fat.

We next assessed how cryopreservation affects adipose-derived stem cell function by culturing the stromal vascular fraction cells from each fat. Adherent cells isolated from both cryopreserved fats grew slowly and occasionally

showed abnormal appearance, which precluded further growth. The number of adipose-derived stem cells cultured from 1 g of fat was lower in cryopreserved fats than in fresh fat. The inferiority of colony-forming scores of adipose-derived stem cells suggests that cryopreserved fats contain fewer functional adipose-derived stem cells than fresh fat.

Finally, we transferred each fat type into immunocompromised mice, to see the true fate of cryopreserved fat grafting. Three months after transplantation, normalized sample weights of cryo-1 and cryo-2 fat were significantly lighter than fresh fat, but more than half the weight of fresh fat for both back and scalp injections. The volume retention of the cryopreserved fat grafts was moderate, which may explain why cryopreserved fat grafting has been accepted by some clinicians. However, both cryopreserved fats were dominated by oil drops surrounded by macrophages, abundant fibrosis, and few viable adipocytes, whereas fresh fat consisted mostly of healthy adipocytes. The presence of oil drops presents a risk of serious clinical complications such as oil cyst formation, chronic inflammation, infection, calcification, and capsular contractures.[6]

Some researchers have suggested that cryopreserved lipoaspirates could be a good method for storing adipose-derived cells.[35] We isolated some functional adipose-derived stem cells from cryo-1 and cryo-2 fats. Moreover, immunohistology of cryopreserved fats 3 months after grafting into mice revealed some small adipocytes, which may be newly regenerated by surviving adipose-derived stem cells. These results support the possibility of cryopreserving adipose tissue for storing adipose-derived cells; however, the method is inefficient, as only a very limited number of adipose-derived stem cells were obtained from the storage.

A limitation of this study is that we evaluated only two methods of preparing cryopreserved fat. Our results suggest that regardless of cooling speed, temperature, and the presence of cryoprotective agents, cryopreservation produces adipose tissues unsuitable for clinical use. However, further optimization of cryopreservation methods and technological advancement may improve results. Alternatively, adjuvant therapy to enhance fat grafting may be beneficial; some researchers reported that cryopreserved fat mixed with culture-expanded human adipose-derived stem cells showed better graft volume and reduced inflammation and fibrosis.[36]

CONCLUSIONS

Our results indicated that most of the adipocytes are killed in the process of cryopreservation and thawing. Adipose-derived stem cells were isolated from cryopreserved fats, but the number of functional adipose-derived stem cells was very limited in both cryopreservation methods. Although clinical volume retention of cryopreserved fat grafting may not differ greatly from that of fresh fat, the grafts remained as dead tissue, including oil cysts and fibrous tissue, which present a risk of serious complications. Thus, cryopreservation of excess harvested lipoaspirates has limited clinical value as far as current technologies are concerned. Future optimization of the cryopreservation protocol may partly solve these problems and work for preservation of adipose-derived stem cells in the lipoaspirates when immediate cell isolation is impossible.

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