OPEN

PRS GLOBAL OPEN

ORIGINAL ARTICLE
Cosmetic

Serial Injections of Cryopreserved Fat at -196°C for Tissue Rejuvenation, Scar Treatment, and Volume Augmentation

Masanori Ohashi, MD
Akihiko Chiba, MD
Hirokazu Nakai, MD
Etsu Fukuda, MD
Takao Higuchi, MD

Background: Fat grafting has become popular since the first report of structural fat grafting in 2001. Fat grafting is effective not only for volume augmentation but also for tissue revitalization. However, fat harvesting is necessary before fat grafting can be performed. Therefore, the performance of serial fat injections is very challenging when treating such patients.
Methods: From August 2015 to March 2017, we investigated 219 patients who underwent fat grafting using the fat that had already been cryopreserved at -196°C.
Results: Follow-up ranged from 3 months to 2 years. No complications occurred, and all outcomes were satisfactory. Three representative cases were also reviewed.
Conclusions: The cryopreserved fat at -196°C could be served as a useful method for serial fat grafting for clinical use; however, further research involving longer follow-up and pathological findings are needed. *(Plast Reconstr Surg Glob Open 2018;6:e1742; doi: 10.1097/GOX.0000000000001742; Published online 18 May 2018.)*

INTRODUCTION

Fat grafting has become popular since Coleman published the first report on structural fat grafting in 2001.[1,2] Many authors have since confirmed the utility of fat grafting in cosmetic and reconstructive surgery,[3–5] because the fat grafting is effective not only for volume augmentation but also for tissue rejuvenation and scar treatment (revitalization/fertilization).[5–7]

The main problem associated with fat grafting for volume augmentation is the unpredictable volume retention rate. Then many idea and devices were invented such as CAL[8]and Brava[9]; however, it may not be enough with only 1 operation.

Therefore, serial injections are needed to reach the ideal volume and obtain an effective outcome.[4,10–12]

Serial injections may also be required for effective revitalization/fertilization of the skin.[4]

Fat harvesting is required to perform fat grafting; however, making serial injection of fat is a substantial challenge for both patients and surgeons. Although many researchers have concluded that cryopreservation of fat is useful under good conditions,[13–15] almost all such studies were experimental. Few articles have described the clinical use of fat cryopreservation.[16,17] In the present study, we ascertained the safety and efficacy of cryopreserved fat at -196°C for clinical use.

PATIENTS AND METHODS

Patients

From August 2015 to March 2017, a total of 455 patients harvested their fat at our clinic and cryopreserved it at -196°C. Table 1 shows these patients' characteristics, including age, height, weight, and body mass index.

Indications

Only patients who provided written informed consent regarding postoperative infection, inflammation, oil cysts, allergy, fat necrosis, and other potential complications were included in the study. Patients younger than 19 years and those with diabetes were excluded.

Anesthesia

Most patients were sedated by intravenous anesthesia and provided local anesthesia by a tumescent technique, but without intubation. No patient was under general anesthesia with intubation during surgery.

From the CLINC Tokyo, Azabu Body Design Center, Tokyo, Japan.

Received for publication August 16, 2017; accepted February 7, 2018.

DOI: 10.1097/GOX.0000000000001742

Disclosure: *The authors have no financial interest to declare in relation to the content of this article. The Article Processing Charge was paid for by the authors.*

Table 1. Patient Characteristics Who Had Cryopreserved their Fat (n = 455)

Duration	August 2015~ March 2017
Sex	Male 30; female 425
Age (y)	19–81 (41.4±11.0)
Height (cm)	142–188 (159.5±9.2)
Weight (kg)	37.7–90.7 (52.4±8.5)
Body mass index (kg/m^2)	16.0–3.3 (20.5±2.6)

Harvesting

Fat was aspirated using a tumescent technique (1 ml of epinephrine, 20 ml of 8.4% sodium hydrogen carbonate, and 50 ml of 1.0% lidocaine per 1,000 ml of saline solution).

Injection on the Same Day of Harvesting (First Injection)

The harvested fat was usually used on the same day as tissue augmentation surgery and/or revitalization/fertilization. In patients who underwent volume augmentation, we basically applied Coleman technique[1,2] (centrifuge 1,200*g*, 3 minutes) and for revitalization/fertilization, we applied nanofat[5]technique (emulsified fat[6]) or squeezed fat.[6]

Sending the Collected Fat

The fat was then sent in the Adiporter to the Cell Processing Center (CPC) at CellSource Corp. in a refrigerated state (below 10°C; Fig. 1).

Compliance/Ethics

In Japan, the Act on the Safety of Regenerative Medicine (Regenerative Medicine Safety Act) came into effect as of November 25, 2014, under an institutional framework for promoting the implementation of regenerative medicine. This act, which covers clinical research and private practice, stipulates 3 risk-dependent standards and the procedures for notification of plans for regenerative medicine as well as the standards of cell culture and processing facilities and the licensing procedures to ensure the safety of regenerative medicine.

In accordance with this law/act, CellSource Corp., Tokyo, Japan (Certification Number: FA3160006) has been certified as CPC by Ministry of Health, Labor and Welfare of Japan.

And fat grafting in the clinic/hospital using the fat that is processed only centrifuged and/or cryopreserved at certified facility is approved in Japan.

Fat Cryopreservation and Storage

The all of fat storage processing were performed following the fat operating procedures by American CryoStem Corp. in the CPC of CellSource Co., Ltd (Tokyo, Japan). In brief, the total fat tissue was washed with enough volume of Ringer's lactate solution and centrifuged at 470 *g* for 3 minutes. The washed fat was rocked with same volume of cryoprotective solution ACSelerate-CP (American CryoStem) for at least 15 minutes. After removal of the excess cryoprotectant, 4–5 mL of the cryoprotectant incorporated fat were transferred to cryovials. The cryovials were cooled at 1°C per minute in a controlled rate freezer to -80°C. Then, for the long-term storage, cryopreserved fat was transferred to the liquid nitrogen tank and stored at -196°C. So, we can make many samples in 1 transportation (Fig. 2).

Recall of the Fat

After we had recalled the required amount of fat through the internet web ordering service of CellSource, the cryopreserved fat was thawed rapidly at 37°C for 6–10 minutes in the CPC of CellSource. After thawing, the fat was washed out cryoprotective agents with flush centrifuge at 470 *g*. The recovered fat was filled in 4 ml syringes and sent to our institution in a refrigerated state (below 10°C).

Stromal Vascular Fraction Count

We counted the number of stromal vascular fraction (SVF) in the cryopreserved and thawed fat before injection in 5 patients. The SVF was isolated by washed and digested by collagenase (Wako Pure Chemical, Osaka, Japan) for 30 minutes at 37°C in a shaking water bath. Cell numbers and viability of SVF were measured with an automated cell counter [LUNA-STEM Automated Fluorescence Cell Counter (Logos Biosystems, South Korea)].

Histological Analysis of Cryopreserved Fat

The returned fat tissues obtained from the same donor were fixed with 10% formaldehyde for histological analysis with H&E staining.

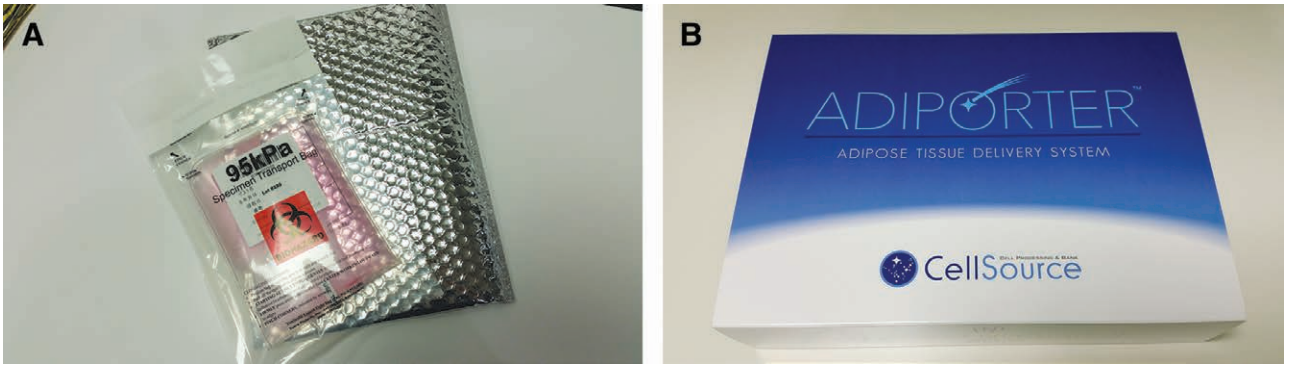

Fig. 1. Transport materials. A, FB-bag (CellSource Corp., Tokyo, Japan), which contains an adipose tissue transport medium (ACSelerate-TR; American CryoStem, Eatontown, N.J.). B, Adiporter (CellSource Corp.) that is the box kit for transportation. The fat was sent in the Adiporter to the CPC at CellSource Corp. in a refrigerated state (below 10°C).

Fig. 2. The collected fat is divided into 4-ml syringes at CellSource Corp. like this picture. So they can make many samples in 1 transportation. This picture was taken after cryopreserved and thawed and returned our clinic. It looks like fresh fat, and there is almost no oil in those syringes.

Injection of Cryopreserved Fat

When we received the fat, we injected it as soon as possible, ideally within 48 hours of being sent to our institution. As for the first injection, we basically applied the Coleman technique, and for revitalization/fertilization, we applied the nonfat (emulsified) technique. The same body parts were not necessarily injected; for example, the first injection may have involved the forehead, whereas the second involved the hands.

Repeat Injections

We recalled the cryopreserved fat again through the internet service of CellSource Corp. if residual fat was present. This system allows for serial fat injections for treatment.

Follow-up

The patients were followed up by physical examination within 1 month and after 6 months postoperatively. And as for breast augmentation, ultrasonography was examined additionally.

RESULTS

The cryopreserved fat was used for the treatment of 219 patients. Table 2 shows the characteristics of the patients who received the cryopreserved fat, including age, height, weight, and body mass index. Table 3 shows the number of injections per person. Table 4 shows the ways in which the cryopreserved fat was used. The injection volume ranged from 0.2 to 24.0 ml for the face and from 4.0 to 100.0 ml for other body regions such as the breasts.

No severe complications occurred, such as infection, fat necrosis, or similar conditions through all patients. Only temporary pigmentation occurred in 5 patients, but all these patients had undergone simultaneous percutaneous aponeurotomy along with the fat grafting.

Table 2. Patient Characteristics Who Received their Cryopreserved Fat (n = 219)

Duration	August 2015 ~March 2017
Sex	Male 16; female 185
Age (y)	19–73 (41.8±11.2)
Height (cm)	144–173 (159.3±10.0)
Weight (kg)	40.4–79.8 (52.1±8.6)
Body mass index (kg/m^2)	16.3–31.6 (20.4±2.8)

Table 3. Number of Injection Times Using Cryopreserved Fat Per Same Person

1 time	219 patients
2 times	47 patients
3 times	17 patients
4 times	5 patients
5 times	2 patients
6 times	1 patient

1 Time means first injection was used fresh fat and cryopreserved fat used 1 time. As follows are same, for example, 2 times means first injection was used fresh fat and cryopreserved fat use 2 times.

Table 4. The Ways to Use Their Cryopreserved Fat (Same Patient Might be Injected Many Places and/or Different Aims)

Volume augmentation only	180 patients
Facial rejuvenation	140 cases
Breast augmentation	54 cases
Hip augmentation	5 cases
Revitalization/fertilization	84 patients
Improve skin condition only	54 cases
Volume augmentation + revitalization/fertilization (treatment for scar and fibrous tissue with/without PALF)	30 cases
Face (injury scar, revision of liposuction, acne scar)	20 patients
Revision of liposuction (thigh, abdomen)	6 cases
Revision of SIEF	3 cases
Incision scar (IMF, nipple, axillar)	7 cases

Rigottomy = percutaneous aponeurotomy and lipofilling (PALF).

Table 5. Volume Change of Fat after Cryopreservation

Volume	Not Centrifuged	Centrifuged
Sent volume (ml)	169±72.7	143±67.9
Returned volume (ml)	59±29.2	76±43.9
Volume (%)	34.4±5.5	51.3±9.8

Not centrifuged means only gravity, and centrifuged means 700–1200 *g*, 3 minutes. Returned volume means cryopreserved and thawed fat, which is possible to use in our clinic.

With regard to the returned fat (cryopreserved and thawed fat, which is possible to use in our clinic), the rate that compares to send out volume was 34.4±5.5% if we sent without centrifuge, and 51.3±9.8% if we already centrifuged (Table 5).

The mean number of SVF in the cryopreserved (returned) fat was 14.8×10^5/ml compared with 7.1×10^5/ml of sent fat (n = 5), so returned fat contains about double amount of SVF.

About histological analysis of cryopreserved (returned) fat, before and after tissues were found to be very similar (Fig. 3).

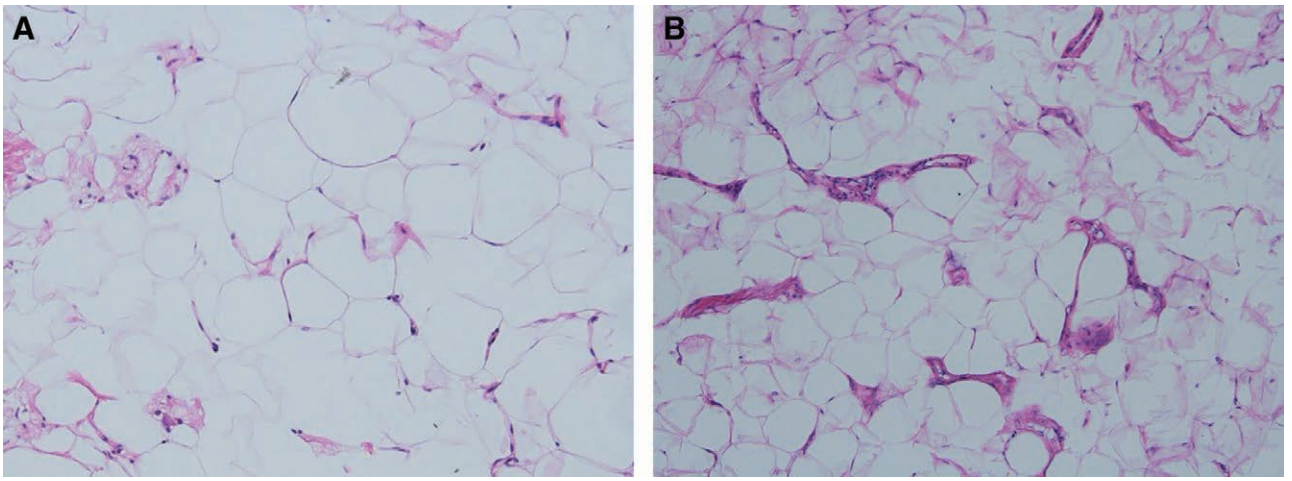

Fig. 3. Histological analysis of the fat tissue before and after cryopreserved and thawed. The fat tissues obtained from the same donor were fixed with 10% formaldehyde for histological analysis with H&E staining (×200). A, The tissue that processed without freezing. B, The tissue that cryopreserved at -196ºC and thawed. The both tissues were found to be very similar.

REPRESENTATIVE CASES

Case 1: Facial Rejuvenation with Fat Grafting by Serial Injections

A 46-year-old woman reported that she was bothered by her thin and aging face (Fig. 4). She underwent facial rejuvenation surgery involving fat grafting of the forehead, cheeks, and lips with a thread lift (Silhouette Soft; Sinclair Pharma, London, United Kingdom). She was concerned about the down-time involved with liposuction, and she cryopreserved her fat at -196ºC. After her first injection, she underwent 2 cryopreserved fat grafting sessions in about 1 year. She appeared younger and healthy after these treatments.

Case 2: Percutaneous Aponeurotomy and Lipofilling[4] by Serial Injections

A 41-year-old woman had undergone simultaneous implant exchange with fat (SIEF; Fig. 5).[18,19] Three months later, her right-side residual capsule was severely shrunken and deformed. Therefore, we harvested her fat again and performed percutaneous aponeurotomy and lipofilling (twice fresh, three times cryopreserved). After the serial injections, the appearance of her breast was very natural.

Case 3: Hand Rejuvenation Using Residual Fat

A 65-year-old woman had undergone breast augmentation with fat grafting and cryopreserved residual fat on the same day (Fig. 6). Four months after the first operation, she underwent hand rejuvenation surgery with her cryopreserved fat. She was thus able to rejuvenate her hands without harvesting.

DISCUSSION

Applications of fat include not only volume augmentation of body parts such as the breasts and gluteals, but also treatment of fibrous and scar tissue such as that affected by scar contracture or radiation damage.[6,20,21]

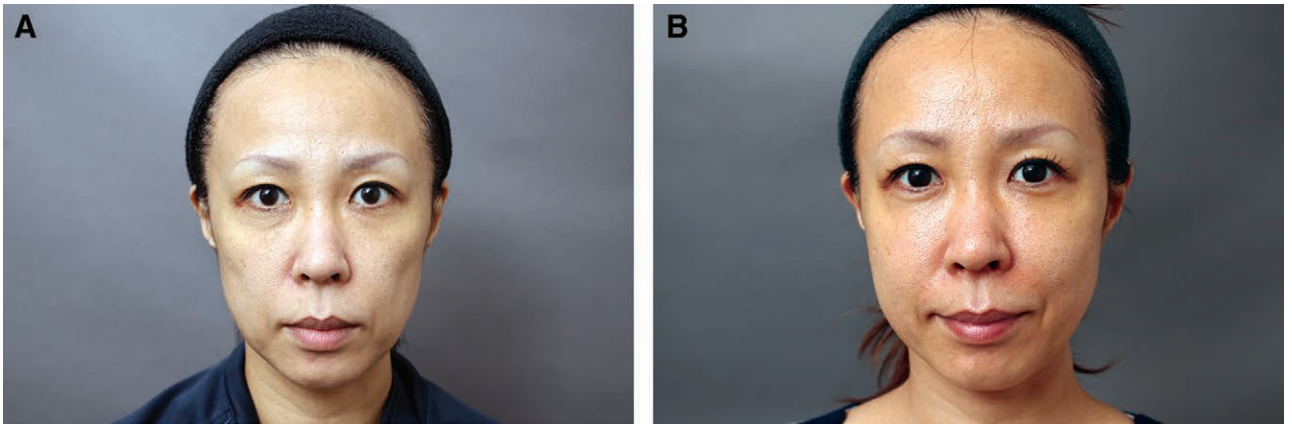

Fig. 4. Case 1: Facial rejuvenation with fat grafting (serial injection). A 46-year-old female received 1 fresh fat grafting (fore head, lower eye lids, cheeks, and lips) with thread-lift (Silhouette Soft, Sinclair, London, United Kingdom). After her first injection, she received 3 times cryopreserved fat-grafting to her forehead, lower eye lids, cheeks, and lips. A, Preoperation. B, 6 Months after second cryopreserved fat grafting.

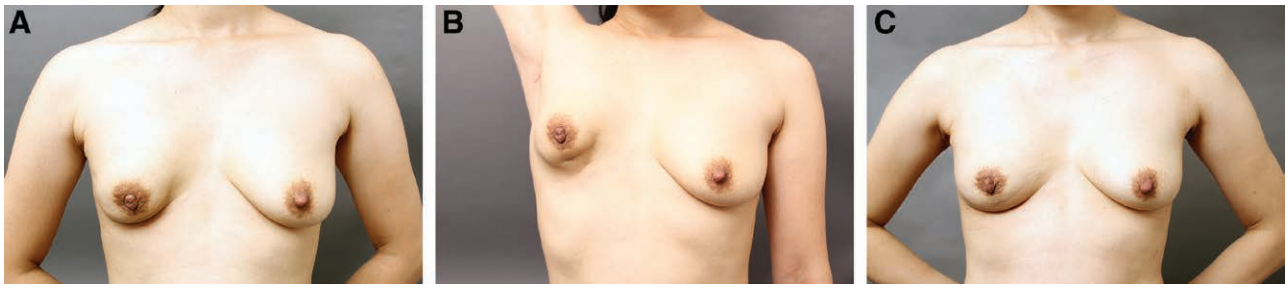

Fig. 5. Case 2: Percutaneous aponeurotomy with fat grafting (serial injections). A 41-year-old female received SIEF operation. A, After 3 months postoperation follow-up. Her right side residual capsule was severely shrunk. B, The picture when she holds up her right arm. C, After third percutaneous aponeurotomy with fat grafting (twice fresh, 3 times cryopreserved).

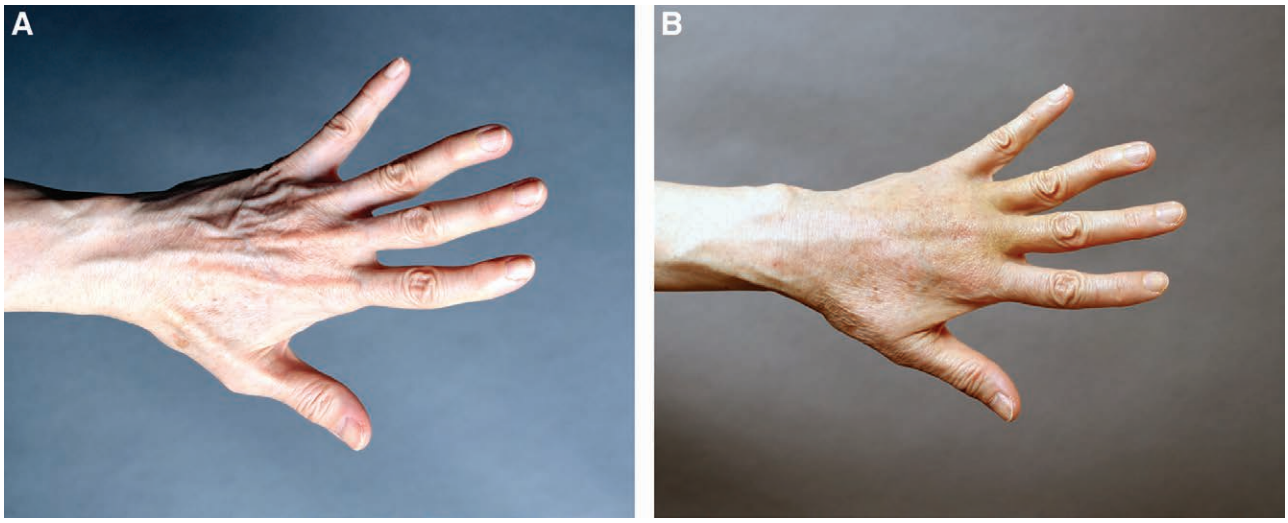

Fig. 6. Case 3: Hand rejuvenation (residual fat use). A 65-year-old female received breast augmentation with fat grafting and cryopreserved residual fat at the same day. After 4 months of first operation, she received hand rejuvenation surgery with her cryopreserved fat. A, Before operation, she did not like her big veins of back of the hand. B, After injected cryopreserved fat to the hand. Veins covered with fat, and unremarkable.

Obstacles to fat grafting include the unpredictable volume maintenance rate for volume augmentation and the unpredictable number of treatments needed to obtain a satisfactory revitalization/fertilization effect. Therefore, many patients need repeat sessions.

However, serial fat grafting with fresh fat imposes a burden on the patient not only their pain but also the medical bill, because harvesting fat is not covered by Japanese health insurance. If we can preserve the fat, we can reduce those burdens.

Thus, plastic/cosmetic surgeons and patients have expressed a strong desire to preserve adipose tissue.

On the other hand, as for the cost of clinic side (specially for small clinic), to preserve the fat, -196°C might be difficult because of high cost due to making CPC and purchasing expensive equipment such as program-freezer.

So, we chose the way that we send the fat to the specialized company and the company cryopreserves the fat in their CPC. Then, small clinic can preserve the fat without high initial investment and maintenance cost. For example, our present protocol involved sending the harvested fat to CellSource Corp. in Tokyo, Japan, and they cryopreserved the fat in their CPC. This system made it easy for us to perform safer preservation without technical or financial difficulties.

Our cases have shown that this method, in which the fat is sent to an external company that cryopreserves it with an adequate method and then we recall the fat, works well for serial injection (cases 1 and 2) and is a useful way to utilize residual fat (case 3). Thus, we believe that this is a very easy method of serial fat grafting and use of residual fat, even in small clinics.

Concerning the cryopreservation of fat, it has been controversial because of viability and safety concerns.[22–24]

Many authors recently suggested that if we use an adequate cryopreservation technique, high fat viability can be achieved.[15,22–26]

The great concerns during cryopreservation are freezing temperature, cooling and thawing temperature, and the use of cryoprotective agents.[22–26]

About freezing temperature, MacRae et al.[25] confirmed that cell frozen at -196°C were less viable that cells frozen at -20°C. Pu[26] remarked that the longer the storage time and the higher the temperature of storage, the less viable

the adipocytes became. About cooling and thawing temperature, cryopreservation cause damage due to intracellular ice formation and osmotic stress. Ice formation can be prevented by controlled slow freezing (1–2°C/min) and rapidly thawing in a 37°C water bath.[27]

And regarding cryoprotective agents, Shu et al.[28] reported the importance of adding cryoprotective agents for better cryopreservation.

Accordingly, we shoe the method that is cryopreserving at -196°C (below -85°C) with the addition of cryoprotective agents, together with controlled slow freezing (1–2°C/min) by program freezer, and rapidly thawing in a 37°C water bath.

Our present protocol involved sending the harvested fat to CellSource Corp. in Tokyo, Japan, and they cryopreserved the fat in their CPC. This made it easy for us to perform safer preservation without technical or financial difficulties (for example, to make CPC).

Our cases have shown that this method, in which the fat is sent to an external company that cryopreserves it with an adequate method and then we recall the fat, works well for serial injection (cases 1 and 2) and is a useful way to utilize residual fat (case 3). Thus, we believe that this is a very easy method of serial fat grafting and use of residual fat, even in small clinics.

Adipocytes have a weaker response to stresses such as ischemia, transportation (mechanical damage),[6] and cryopreservation than do adipose-derived stem cells.

As our result, the volume of the fat returned to our clinic is smaller than that sent out from our clinic. This is 1 of the disadvantages of this system.

On the other hand, the mean number of SVF in the cryopreserved (returned) fat was 14.8×10^5/ml compared with 7.1×10^5/ml of sent fat (n = 5). So returned fat contains about double amount of SVF. Thus, cryopreservation could be considered as an option for condensing adipocyte-derived stem cells.

In any case, with our present protocol, the amount of adipocyte and SVF were decreased through the process of transportation, freezing, and thawing. So we should improve our protocol and technique of cryopreservation for cell damages.

However, the fact that no complications occurred among all 219 patients indicates the safety of serial injection using cryopreserved fat, at least in the short-term follow-up. We did not compare the retention rate and effect of revitalization/fertilization with those of fresh fat. Further research involving longer follow-up is needed to determine whether cryopreserved fat can serve as a new option for serial fat grafting.

Masanori Ohashi, MD
THE CLINC Tokyo
Azabu Body Design Center 1F
3-16-23 Nishiazabu, Minato-Ku
Tokyo 106-0031, Japan
E-mail: ohashi0822@gmail.com

ACKNOWLEDGMENT

The authors thank Hideto Kaneshima, MD, PhD, Satoshi Tsunoda, and Syunsuke Tazumi from CellSource Corp. for providing the method of cryopreserving and thawing. The authors also thank Yusuke Shimizu, MD, PhD, who is a professor of Department of Plastic and Reconstructive Surgery in University of the Ryukyus Hospital, Okinawa, Japan, for giving many ideas.

REFERENCES

1. Coleman SR. Structural fat grafts: the ideal filler? *Clin Plast Surg.* 2001;28:111–119.
2. Coleman SR. Structural fat grafting: more than a permanent filler. *Plast Reconstr Surg.* 2006;118:108S–120S.
3. Khouri RK, Rigotti G, Cardoso E, et al. Megavolume autologous fat transfer: part I. Theory and principles. *Plast Reconstr Surg.* 2014;133:550–557.
4. Khouri RK, Smit JM, Cardoso E, et al. Percutaneous aponeurotomy and lipofilling: a regenerative alternative to flap reconstruction? *Plast Reconstr Surg.* 2013;132:1280–1290.
5. Tonnard P, Verpaele A, Peeters G, et al. Nanofat grafting: basic research and clinical applications. *Plast Reconstr Surg.* 2013;132:1017–1026.
6. Mashiko T, Wu SH, Feng J, et al. Mechanical micronization of lipoaspirates: squeeze and emulsification techniques. *Plast Reconstr Surg.* 2017;139:79–90.
7. Sautereau N, Daumas A, Truillet R, et al. Efficacy of autologous microfat graft on facial handicap in systemic sclerosis patients. *Plast Reconstr Surg Glob Open.* 2016;4:e660.
8. Yoshimura K, Sato K, Aoi N, et al. Cell-assisted lipotransfer for cosmetic breast augmentation: supportive use of adipose-derived stem/stromal cells. *Aesthetic Plast Surg.* 2008;32:48–55; discussion 56.
9. Khouri RK, Rigotti G, Khouri RK Jr, et al. Tissue-engineered breast reconstruction with Brava-assisted fat grafting: a 7-year, 488-patient, multicenter experience. *Plast Reconstr Surg.* 2015;135:643–658.
10. Losken A, Pinell XA, Sikoro K, et al. Autologous fat grafting in secondary breast reconstruction. *Ann Plast Surg.* 2011;66:518–522.
11. Gatti JE. Permanent lip augmentation with serial fat grafting. *Ann Plast Surg.* 1999;42:376–380.
12. Kim HY, Jung BK, Lew DH, et al. Autologous fat graft in the reconstructed breast: fat absorption rate and safety based on sonographic identification. *Arch Plast Surg.* 2014;41:740–747.
13. Pu LL, Coleman SR, Cui X, et al. Cryopreservation of autologous fat grafts harvested with the Coleman technique. *Ann Plast Surg.* 2010;64:333–337.
14. Gir P, Brown SA, Oni G, et al. Fat grafting: evidence-based review on autologous fat harvesting, processing, reinjection, and storage. *Plast Reconstr Surg.* 2012;130:249–258.
15. Pu LL, Cui X, Fink BF, et al. Adipose aspirates as a source for human processed lipoaspirate cells after optimal cryopreservation. *Plast Reconstr Surg.* 2006;117:1845–1850.
16. Ibrahiem SMS, Farouk A, Salem IM. Facial rejuvenation: serial fat graft transfer. *Alexandria J Med.* 2016;52:371–376.
17. Butterwick KJ, Bevin AA, Iyer S. Fat transplantation using fresh versus frozen fat: a side-by-side two-hand comparison pilot study. *Dermatol Surg.* 2006;32:640–644.
18. Del Vecchio DA. "SIEF"—simultaneous implant exchange with fat: a new option in revision breast implant surgery. *Plast Reconstr Surg.* 2012;130:1187–1196.
19. Ohashi M, Yamakawa M, Chiba A, et al. Our experience with 131 cases of simultaneous breast implant exchange with fat (SIEF). *Plast Reconstr Surg Glob Open.* 2016;4:e691.
20. Khouri RK Jr, Khouri RK. Current clinical applications of fat grafting. *Plast Reconstr Surg.* 2017;140:466e–486e.
21. Rigotti G, Marchi A, Galiè M, et al. Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing pro-

cess mediated by adipose-derived adult stem cells. *Plast Reconstr Surg.* 2007;119:1409–1422; discussion 1423.
22. Pu LL, Cui X, Fink BF, et al. Long-term preservation of adipose aspirates after conventional lipoplasty. *Aesthet Surg J.* 2004;24:536–541.
23. Wolter TP, von Heimburg D, Stoffels I, et al. Cryopreservation of mature human adipocytes: *in vitro* measurement of viability. *Ann Plast Surg.* 2005;55:408–413.
24. Jeon IK, Lee H, Shin JY, et al. Cryopreserved autologous fat injections as a filler agent for facial augmentation: are they still safe? *Yonsei Med J.* 2014;55:280–281.
25. MacRae JW, Tholpady SS, Ogle RC, et al. Ex vivo fat graft preservation: effects and implications of cryopreservation. *Ann Plast Surg.* 2004;52:281–282; discussion 283.
26. Pu LL. Cryopreservation of adipose tissue. *Organogenesis.* 2009;5:138–142.
27. Hwang SM, Lee JS, Kim HI, et al. Comparison of the viability of cryopreserved fat tissue in accordance with the thawing temperature. *Arch Plast Surg.* 2015;42:143–149. doi: 10.5999/aps.2015.42.2.143.
28. Shu Z, Gao D, Pu LL. Update on cryopreservation of adipose tissue and adipose-derived stem cells. *Clin Plast Surg.* 2015;42:209–218.