


Cryopreservation of lipoaspirates: in vitro measurement of the viability of adipose-derived stem cell and lipid peroxidation

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Abstract

As the storage time of the fat tissue passes by, lipid peroxidation and creation of by-products may take place. The objective of this study was to evaluate the cell viability and functional changes of adipose-derived stem cells (ADSCs) in the cryopreserved lipoaspirates at different temperatures in accordance with lipid peroxidation. Lipoaspirates acquired from liposuction were divided into four different temperature groups and stored at 4°C, -20°C, -80°C, and -196°C. After isolating ADSC from each sample, gross cell morphology and cell viability were compared with doubling time and colony-forming unit (CFU) formation ability. Acid value, that is, thiobarbituric acid value was measured to assess lipid peroxidation. No viable ADSC was observed in -20°C and -196°C samples for past 1 week and a superior number of the live cells were detected in the 4°C group compared with the -80°C group. However, the persistence of cell division and CFU formation after 1 week was only observed in adipocytes stored at -80°C. Lipid peroxidation mainly occurred at 4°C and -20°C storage samples. If the lipoaspirates were planned to be cryopreserved, it is advised to store at -80°C. However, the number of actually functional ADSCs is very low. Furthermore, even in the cryopreserved status, continuous lipid peroxidation and by-product creation took place, suggesting shorter preservation period as possible in the clinics.

KEYWORDS

adipose-derived stem cell, cryopreservation, fat graft, lipid peroxidation, liposuction

1 | INTRODUCTION

The main drawback of autologous fat graft is its low predictability because of absorption or necrosis of transplanted adipose tissue. The variable rate of autologous fat graft survival, ranging from 30% to 80%, has been

reported.¹⁻³ Clinically, progressive fat resorption takes place for 3 months after surgery and shows stable integration only after this period.^{4,5}

Therefore, fat grafting usually requires repetitive injections to achieve reconstructive or aesthetic treatment goals. To avoid multiple harvesting procedures,

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cryopreservation and subsequent use of remnant lipoaspirate have been practiced by some clinicians without definite supporting evidence. Reported storage time of the frozen lipoaspirate is up to 7 years.⁶ However, it was revealed that as most of the adipocytes are killed in the process of cryopreservation and thawing, the volume preservation of cryopreserved fat is lower than fresh fat and these necrotic tissues may increase the risk of serious complications, such as oil cyst formation, chronic inflammation, and progressive calcification.^{5,7,8}

There have been a number of studies to develop a better technique for long-term preservation of fat grafts. Some consensus is forming on freezing and thawing temperatures and protocols and the use of cryoprotective agents (CPAs).^{9,10} Nevertheless, there was no definite study on progressive chemical changes of the adipose tissues other than cryodamage during first 3 months of preservation period. In the field of sitology, among the cryopreserved nutrients, fat is known to undergo lipid peroxidation (LPO) (or rancidity).¹¹⁻¹³ More relevant example of LPO is occurring at cryopreservation of semen (or sperm) for artificial insemination.¹⁴⁻¹⁷ The authors hypothesize that cryopreserved fat tissue may undergo similar change in some extent. Here, we investigated the evidence of LPO and consequent effect on cell viability that occurs during 3 months in cryopreserved adipose tissue at four different clinically accessible storage temperatures.

2 | MATERIALS AND METHODS

2.1 | Harvesting lipoaspirate

Fat tissue was harvested from one healthy female patient who underwent conventional liposuction of the abdomen or thigh. Before the harvest, the patient provided her informed consent about the research on human-derived material. The study protocol approval was obtained from The Catholic University of Korea Catholic Medical Center Institutional Review Board (KCMC IRB). The samples were divided into three partitions and each partition was again divided into four temperature storage samples. The following assessment methods were repeated for total 12 samples and the average value was documented.

2.2 | Procedure of cryopreservation (freezing and thawing protocol)

Adipose tissue was harvested by a power-assisted liposuction with 3.0 mm diameter suction cannula and centrifuged at 3000 rpm for 3 minutes. After discarding the supernatant lipid layer and the bottom plasma and

Key Messages

- Only lipoaspirates stored in -80°C contained viable adipose-derived stem cells (ADSCs) until 84 days. However, absolute number of viable and functional ADSCs was very low
- A greater number of ADSCs in 4°C storage samples survived until 28 days but the functional ability was severely compromised
- Fat preservation in 4°C showed great increase in acid value as time passes. Continuous lipid peroxidation and by-product creation mainly occurred in 4°C and -20°C storage samples
- When considering cryopreservation of lipoaspirate for later clinical or laboratory use, -80°C storage should be favoured within 3 month period

aqueous layer, only the fat containing middle layer was saved. The resulting fat layer was divided into five groups including one "initial fat" group and four preserving groups. Adipose tissues were contained in a sterile plastic container and preserved at 4°C , -20°C , -80°C , and -196°C . A commercial refrigerator (LG, Seoul, Korea) was used for 4°C and -20°C samples and controlled rate freezer (LG, Seoul, Korea) was used for -80°C and -196°C samples for programmed freezing with $1^{\circ}\text{C}/\text{minute}$ freezing steps until -80°C and stored in deep freezer (Thermo Fisher Scientific, Waltham, Massachusetts) at -80°C or liquid nitrogen at -196°C . After 1, 2, 4, 8, and 12 weeks later, the completed samples were retrieved and then thawed for 5 minutes in a 37°C water bath. Gross morphology and free oil formation were checked after thawing. (Figure 1).

2.3 | Acid value

Thawed fat of 1 g was suspended in ethanol (Merck Millipore, Burlington, Massachusetts)-ether (Samchun Chemicals, Seoul, Korea) 2:1 solution and two drops of 1% phenolphthalein solution (Samchun Chemicals) was added. Then, 0.1 N KOH (Sigma Aldrich, St. Louis, Missouri) was titrated until the ethanol-ether solution turn into pink colour. The acid value was calculated as follows.

Acid value (mg KOH/g) = $5.611 \times (A - B) \times F/S$, where A = KOH consumption of the sample (ml), B = KOH consumption of the control (ml), F = Normality of 0.1 N KOH, and S = Weight of sample (g).

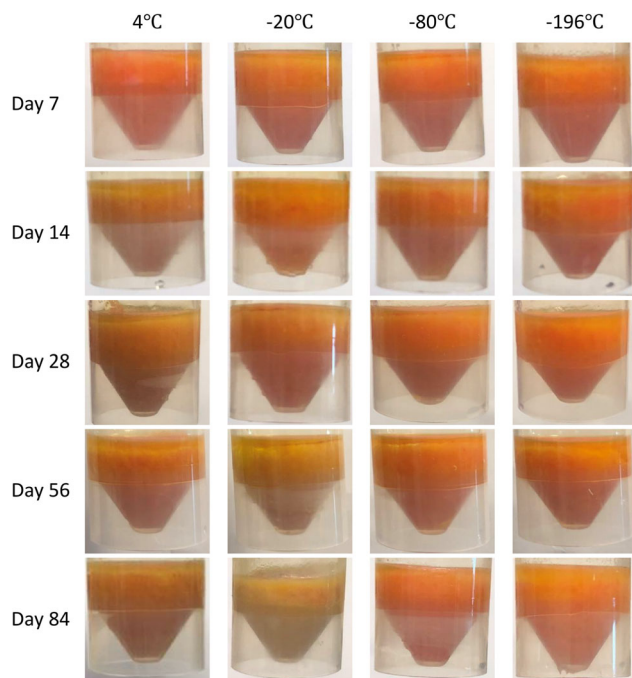


FIGURE 1 Macroscopic appearance of the cryopreserved fat tissue after thawing

2.4 | Lipid peroxidation assay

LPO was measured using the modified thiobarbituric acid assay (TBA) through measuring malondialdehyde. Malonaldehyde (MDA) level was detected by LPO assay kit (cat. no. ab118970, Abcam, Cambridge, UK). Immediately after thawing, 100 mg of fat tissue was vigorously vortexed in 300 μ L MDA lysis buffer and 3 μ L 100X Butylated hydroxytoluene for a minute. After 10 minutes of centrifuge with 13 000g at 4°C, 200 μ L of supernatant was transferred to a new tube and added 600 μ L TBA solution. TBA-sample mixture was incubated at 95°C for 60 minutes then cooled to room temperature in an ice bath for 10 minutes. The mixture was filtered with 0.20- μ m-sized syringe filter (Whatman, Maidstone, UK) and transferred to 96-well microplate and OD (optical density) was measured at 532 nm for MDA level.

2.5 | Isolation and culture of ADSC

The adipose tissue taken by liposuction was dissociated using 0.1% collagenase type I (Sigma Aldrich, St. Louis, Missouri) and cultured at 37°C for 30 minutes in cellticator (Medikan, Seoul, Korea). Subsequently, after neutralising the activated collagenase with 10% fetal bovine serum (Gibco, Grand Island, New York), in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 1% antibiotic/antimycotic (Gibco), the lipoaspirate

was centrifuged for 3 minutes at 1300 rpm, and the upper layer of liquid and free oil layer was removed. Resultant pellet was filtered with 100- μ m-pore-sized strainer (Falcon Corning Inc., New York City, New York) and 3 more minutes of centrifuge at 1300 rpm was carried out. After removing supernatant, separated stromal vascular fraction (SVF) was suspended in new DMEM. Haemocytometer (INCYTO, Cheonan, Korea) was used for cell counting. The cells in SVF were seeded to culture dishes and then cultured at 37°C in a 5% CO₂ incubator for 24 hours. The medium was removed from the dishes 24 hours later. The dead cells were removed by repeatedly cleaning with phosphate-buffered saline (PBS, Gibco). New DMEM was added a week later and the cells were subcultured as "passage 1."

2.6 | Cell viability

The viable adipose-derived stem cells (ADSCs) were counted with Haemocytometer (INCYTO). Trypan blue exclusion method was used to select live cell. After 0.4% trypan blue vital stain (Gibco) of centrifuged cell pellet, the number of viable adipocytes was then counted with a haemocytometer under a microscope with $\times 40$ magnification. A live cell was not stained by trypan blue, whereas a dead cell was dyed blue. The cell viability was calculated by dividing the number of live cells with initial total (live + dead) cell number (Cell viability (%) = live cell count ($\times 10^6$ /g)/initial total cell count ($\times 10^6$ /g) $\times 100$).

2.7 | Doubling time and CFU-F assay

To assess function of ADSC, doubling time and colony-forming unit fibroblast (CFU-F) assay was used.

Doubling time of ADSC was measured with EZ-Cytox (cat. no. EZ-3000, DoGen, Seoul, Korea). Cells of passage 1 were seeded to 96-well microplate with concentration of 3000 cells/well and cultured at 37°C in a 5% CO₂ incubator for 3 days; two more hours in 5% CO₂, 37°C incubator after changing the solution to 1:9 EZ-Cytox: DMEM solution. Then, the supernatant was transferred to new 96-well microplate, and 450 nm absorbance was measured.

CFU-F assay was carried out with cells of passage 1 seeded to 1000 cells/100-mm culture plate which were incubated in 5% CO₂, 37°C incubator for 3 weeks. Three weeks later, culture medium was removed and washed with PBS three times. Then, cell fixation was carried out with methanol (Duksan, Seoul, Korea). After 5 minutes of fixation, methanol was removed and the staining was

carried out with 0.5% crystal violet (Sigma aldrich) solution. The stained cells were washed several times with PBS and distilled water. After drying in room temperature, the CFUs of ADSC were counted.

2.8 | Statistics

All data in this study were expressed as mean \pm SD. A two-tailed paired Student *t* test was used to assess the difference between the two groups. A *P*-value less than .05 was considered statistically significant.

3 | RESULTS

3.1 | Gross morphology

The extent of adipocyte death from freezing and thawing may be estimated by free oil formation. The oil released from thawed adipose tissue was not conspicuously different with time but the samples frozen in -196°C showed more oil droplets in smear slides. (Data S1) The sample stored in 4°C showed gradual increase of free fat and colour change to light yellow. It is

suggested that adipocyte damage is mainly from freezing and thawing process itself, more prominent at -196°C , and relatively active metabolic rate in 4°C might cause harmful metabolites for adipocytes over time, causing cell damage (Figure 1).

3.2 | Acid value

Acid value indicates the mass of potassium hydroxide required to neutralise 1 g of substance. It measures the amount of carboxylic acid groups such as fatty acid. As fats rancidify, triglycerides are converted into fatty acids and glycerol, causing an increase in acid number. Over time, gradual increase in acid value was prominent in 4°C samples. Other lipoaspirates stored in frozen state did not show noticeable changes (Figure 2A).

3.3 | Viability of ADSC (yield)

After extracting SVF from three partitions, the number of ADSCs per gram of lipoaspirate and viability of ADSC was counted. The average cell viability was 96.093%.

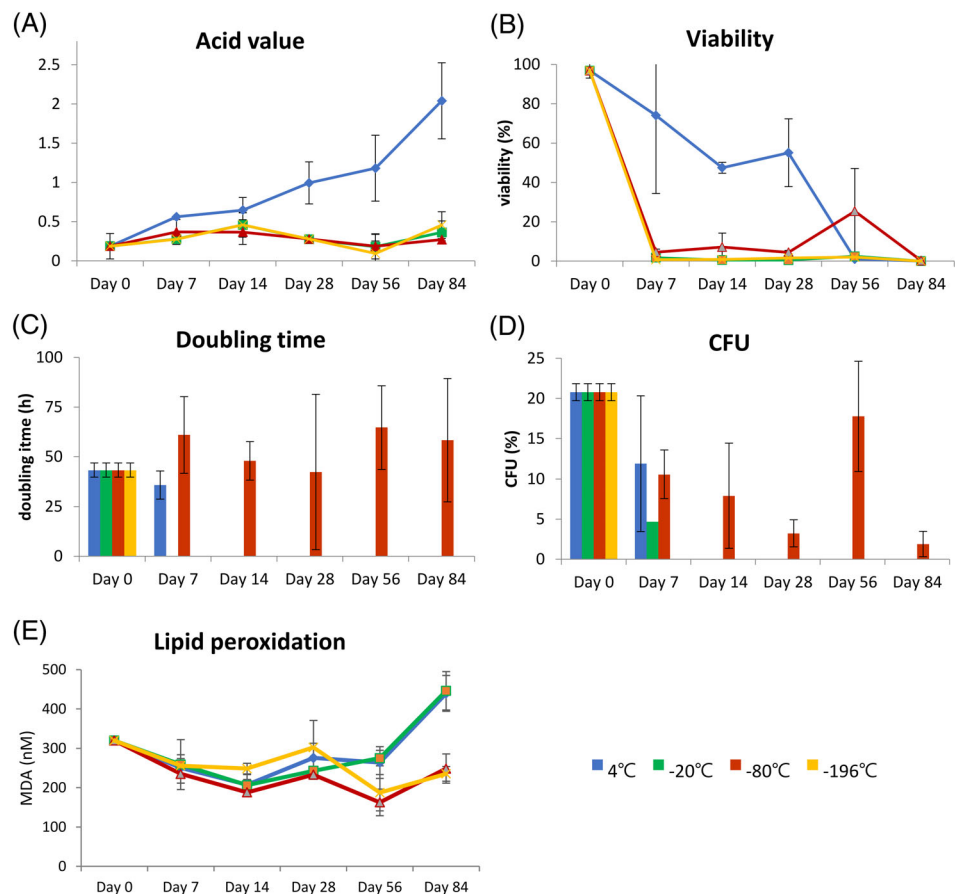


FIGURE 2 The graphical representation of results of four different temperatures. A, Acid values of lipoaspirates. B, Viability of ADSC. C, Doubling time of ADSC. D, Colony-forming unit fibroblast assay result of ADSC. E, Concentrations of Malonaldehyde in samples over time. ADSC, adipose-derived stem cells

The assessment after 1 week of storage revealed drastic decrease in viable cell count in three cryopreserved groups. The viable cells at -20°C and -196°C were barely detectable, while relatively high number of viable cells were detected at -80°C . After 2 weeks, none or only one cell was detected in -20°C and -196°C samples, whereas -80°C sample showed maintenance of the number of viable cells. However, after 84 days, viable cell in -80°C sample was also barely detectable. The samples stored in 4°C showed relatively gradual decrease in viability until 28 days. But no more cells were detectable after 56 days. (Figure 2B).

3.4 | Doubling time

The doubling time of ADSC showed no statistical difference at P0. At day 7, only ADSC from 4°C and -80°C samples showed cell proliferation capacity. After 14 days, only cells from -80°C were able to divide and this characteristic remains until day 84 (Figures 2C and 3).

3.5 | Colony-forming unit fibroblast

CFU formation was detected in all samples at day 0 and day 7. But relatively lower number of CFU was observed. From day 14, only ADSCs from -80°C samples were able

to develop CFU. At day 84, even in -80°C samples, CFU number showed significant drop (Figures 2D and 4).

3.6 | Lipid peroxidation

All groups showed gradual decrease in MDA until day 14. After 2 weeks, MDA concentration in 4°C and -20°C samples began to rise until day 84, whereas -80°C and -196°C samples maintained similar level of MDA until day 84 (Figure 2E).

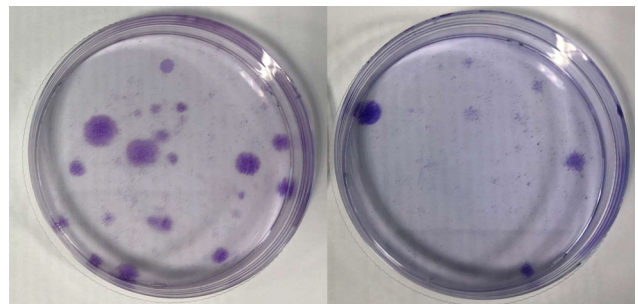


FIGURE 4 Representative photo of colony-forming unit fibroblast assay. (Left) Day 0 fresh fat (20 counts), (Right) Day 28 (three counts)

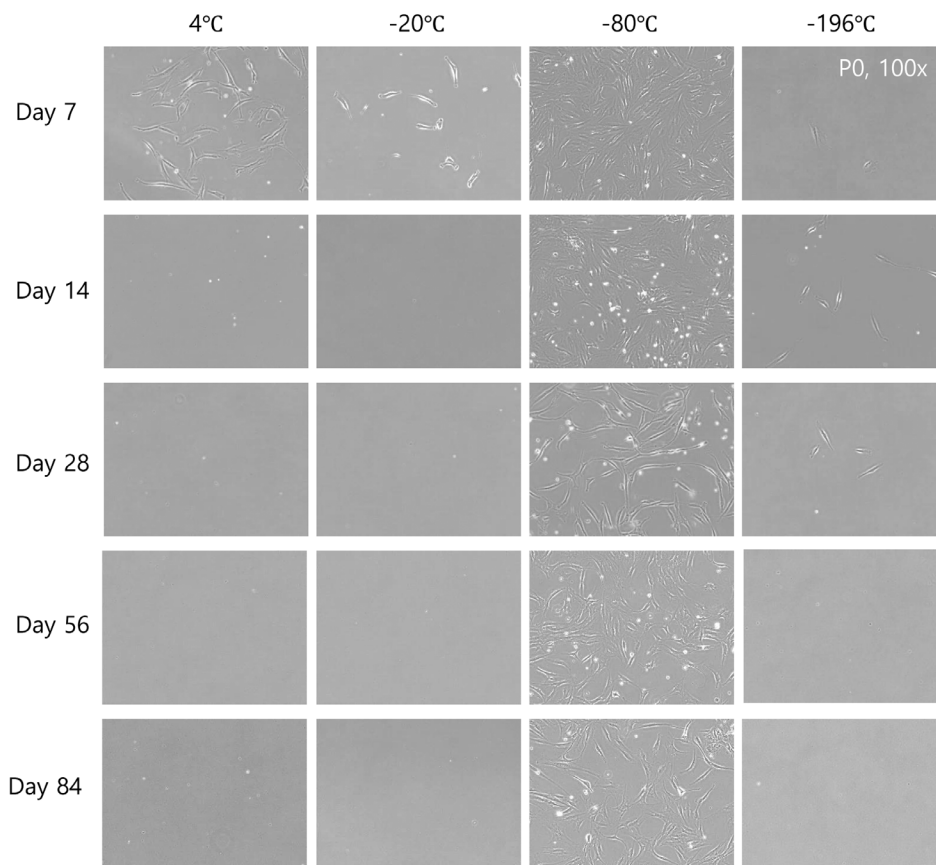


FIGURE 3 Cultured ADSC on microscope. Only ADSC from -80°C sample showed cell proliferation ability, even at day 84 after storage. ADSC, adipose-derived stem cells

4 | DISCUSSION

The main purpose of fat graft is accomplished by its volume retention. The volume of cryopreserved fat graft is decided from a survival rate of cells from the graft and a migration of cells from the recipient site. ADSC composes minor fraction in the graft, but their potential for regeneration is greater than mature adipocytes. Studies of which ADSC or SVF is added to enhance fat graft survival suggest that progenitor cells elicit crucial role in cell proliferation as well as angiogenesis and anti-apoptosis.¹⁸⁻²¹ Therefore, our study focused on the survival and function of ADSC in the cryopreserved lipoaspirate.

Most other studies reported that positive results on cell viability of cryopreserved adipocytes had some methodologic errors or limitations, such as accessing tissues frozen only for a short period of time, viability studies immediately after thawing was carried out, *in vitro* study only, or using an animal fat tissue. There were only few studies evaluating the frozen fat tissue after it has been stored more than a month. Progressive fat resorption is known to be stabilised after 3 months and repeated supplementary fat graft is most needed only after this period.⁴ So, animal or human studies that have shown considerable fat survivals in short term may be less relevant to clinical field. In Son's study, fat tissue stored at -15°C and -70°C and viability were measured until 56 days. The viability of adipocytes rapidly declined after 1 day of cryopreservation and showed gradual decrease until only 5% of the fat cells were alive at 8 weeks. However, Zheng et al grafted cryopreserved tissue (-20°C , -80°C , and -196°C) to a back of nude mice and achieved a relatively good filling effect. This finding was explained as primary filling effect of dead adipocytes serving as good scaffold, containing effective extracellular matrix that supports the growth of surrounding cells. Furthermore, proliferation of few survived stem cells or transformation of exogenous SVF into adipocytes in frozen fat grafts may contribute. Our cell yield was very low after freezing/thawing and the graft was filled with dead cells.²² Clinically, Butterwick et al showed equivalent to improved result of frozen fat graft compared with fresh fat graft by side-by-side two-hand comparison pilot study.²³ These results may delude the meaning of dead cells in fat graft. Unviable adipocyte could act as extracellular matrix filler and induce subclinical inflammation reaction that resulted in positive tissue rejuvenation effects. But histologic examination of cryopreserved fat with high necrotic cell proportion shows high rate of graft absorption as well as oil cyst formation, chronic inflammation, and progressive calcification.²² As compared with other currently available cryogenic banking of various tissues such as skin, heart valve, and dura mater, which the

cryopreserved tissue serves only as tissue matrix scaffold, fat graft requires elaborate strategy to retain its volume without complications. The cell yield in our study was also extremely low, indicating that graft volume maintenance in clinical cryopreserved fat graft is less likely because of adipocyte survival and proliferation.

Numerous studies have investigated fat survival from different cryopreservation techniques. Different freezing temperatures were tried and fat stored at subzero temperatures were shown to be better, because it maintained its mitochondrial metabolic activity up to 8 days.^{24,25} Comparison between -20°C , -80°C , and -196°C showed no significant difference in cell viability for 7 days of cryopreservation in one study, but more viable cells were found in tissues stored at -80°C than at -20°C in other study.²⁶⁻²⁸ Gao et al suggest that once the fat grafts are frozen in liquid nitrogen at -196°C , the living cells within the fat grafts are simply "sleeping" with little metabolism and structural changes and thus can be stored as long as they are needed.²⁹ However, as himself mentioned, most adipocytes were killed in the process of cryopreservation and thawing. Furthermore, if a "tissue" is cryopreserved, mechanical damage from the intracellular/extracellular ice crystals and the increase in concentration of solutes easily occur, because of difficulty in diffusion of any innate/infused cryoprotectant into each cell.³⁰ The method of freezing and thawing may alter proportions of live cells. However, in general, nevertheless about 66.2% to 77% of cell necrosis is inevitable in all subzero samples; controlled freezing and rapid thawing method is gaining consensus as a most effective method.^{3,25,29,31} Especially without any CPAs, fat tissues that traverse through -196°C undergo devastating changes and produce significantly more free oil droplets compared with -20°C and -80°C samples as in our study.^{5,22} Storing fat tissue at liquid nitrogen requires a complex facility, so liquid nitrogen is limited in clinics. However, by using commercial freezers, cryopreservation at -20°C or -80°C may be accessible for surgeons to cryopreserve fat tissue effectively, so we focused on comparing the effects of cryopreservation in those temperatures.²⁷ Consistent with other studies, cryopreservation in -80°C was most effective for 3 months of lipoaspirate cryopreservation compared with -20°C or -196°C in terms of cell viability and proliferation ability.^{5,22,26,27}

Zheng et al revealed that superior amount of SVF was harvested in fat that was stored for 1 week in 4°C compared with frozen fat samples, suggesting that 4°C cryopreservation temperature is beneficial for short-term storage.²² Matsumoto et al compared cell viability of adipose tissue in different storage temperatures and observed that preservation for 24 hours at 4°C shows similar yield of adipose-derived stem cell to that of fresh aspirated fat.

However, yield drops to uncertain level after 2 or 3 days.²⁸ Our result also showed superior cell survival at 4°C until 28 days of preservation. This may be because of absence of cell damages from freezing/thawing and low metabolic activity. However, after 1 week, thought-to-be-viable cells of 4°C samples lose their ability to proliferate. As Pu et al suggested, we hypothesized that cryopreserved cells have difficulty in resuming optimal intracellular enzyme activity for cell survival or proliferation.³² A reasonable doubt arose that intracellular formation and accumulation of certain metabolites may cause these irreversible changes as it tends to progress with time. Evanelista-Vargas et al investigated intracellular superoxide anion and hydrogen peroxide changes during cooling curve at 25°C, 15°C, 5°C/0 min, 5°C/15 min, and 5°C/30 min and after freezing/thawing of alpaca spermatozoa. Maximal percentage of total spermatozoa producing superoxide anion and hydrogen peroxide (H₂O₂) were found at 5°C/30 min. Interestingly, after thawing, the superoxide anion and hydrogen peroxide levels returned to baseline values.¹⁷ Superoxide anion and H₂O₂ in the fat preserved at 4°C may maintain in higher level causing continuous oxidative stress. Because the metabolic activity is low and not absent in 4°C, by-product accumulation with consequent innate antioxidant depletion leads to cell damage and explains exponential rise of the acid value, gradual cell deterioration, and failing to survive. Taken together, in 4°C, only 24 to 48 hours of preservation is valid for re-grafting or ADSC extraction.

Fat cell contains triglyceride and fatty acid. When fat or oil is exposed to air with/without light (oxidative), moisture (hydrolytic), or bacterial action (microbial), complete or incomplete oxidation or hydrolysis takes place, resulting in short chain aldehydes and ketones. This is called rancidification, and oxidative rancidification is known to take place even in a frozen state. LPO is oxidative degradation of lipids where the double bond of an unsaturated fatty acid is cleaved by free radical reactions producing H₂O₂ and various aldehydes and ketones. MDA is one of the final products, and by using TBA method, the level of MDA is measured to estimate the extent of LPO. In cryopreservation of sperm or semen, which is common procedure than fat cryopreservation, many studies demonstrate increase in reactive oxygen species (ROS) production with LPO during the process of cryopreservation.^{14-17,33} The plasma membrane of spermatozoa contains polyunsaturated fatty acids which bind with oxygen and produce high level of ROS. The semen endows endogenous antioxidant system, but if the ROS generated from dead cells exceed the limit of ROS elimination, accumulation of oxidative stress take place. In consequence, functional and structural integrity of membranes breaks down, resulting membrane permeability

deterioration, DNA damage, and eventually cell death. Sperm is known to be more durable than tissue in cryopreservation, although recent studies revealed that cell viability and activity (presented as fertility) are affected by intracellular LPO and ROS formation in long-term preservation. In fat cryopreservation, the same concept may take into consideration, as LPO of adipocyte or other cells in fat tissue occurs, ROS formation and LPO may affect cell viability. However, the H₂O₂ level did not show any significant or uniform changes probably because of, as mentioned earlier, regression of the H₂O₂ level after thawing. This may be explained as recovery of antioxidant capacity of enzymes increased with the thawing temperature and because of short half-life of ROS, oxygen radicals capable of destroying nucleic acids generated during the thawing process is mostly decomposed after thawing.^{14,34} The samples were tested after thawing; therefore, measuring the H₂O₂ cannot be a representative value for accumulative cell damage. However, MDA level showed gradual decrease for the first 2 weeks and conversion to gradual increase between second and eighth week. One possible interpretation is that the stock of scavenger system may be ample to withstand LPO in the first 2 weeks. As cryopreservation continued for past 2 weeks, the environment with more oxidative stress begin to be depleted with antioxidants; therefore, LPO take place. Therefore, increase of MDA level between day 56 and day 84 of 4°C and -20°C samples was observed. Further study with adding or depleting antioxidant before cryopreservation may make the matter clear.

Using CPAs, such as dimethyl sulfoxide (DMSO) and glycerol, is another issue. Both were known to be effective in preventing cell damage from cryopreservation, but DMSO is cytotoxic chemical which may not be used clinically, and glycerol requires repeated washing steps before grafting, which also has some drawbacks in actual clinical use. Hydroxyethyl starch (HES) is a non-membrane-permeating CPA which causes less toxicity to cells and therefore is an ideal agent for clinical use, but the study of Li et al and Wolter et al reported no significant cell protecting effect between the HES and the control groups.^{26,27,33}

Cell survival generally can be defined in terms of its viability and/or its ability to grow and develop. The former is mainly carried out with cell membrane integrity assays, whereas the latter is evaluated by functional assays such as MTT, XTT, and acid phosphatase assays. However, in fat graft, the main goal is maintenance of its volume and graft volume loss is thought to be compensated by ADSC which comprise less than 1% of lipoaspirate.³⁵ Therefore, functional assay of adipocyte viability in fat graft should be focused on cell proliferation ability rather than examining mitochondrial enzyme

function. In our study, viable cell count was carried out with trypan blue staining, one of which methods identifies cell membrane integrity to access cell viability. The possibility of DNA damage and non-functional cell with intact cell membrane at the time of the measurement cannot be excluded. Cells are not simply bags of cytoplasm bound by a plasma membrane.²⁹ So as to complement analysis of trypan blue staining, we performed doubling time and CFU formation assessment. One interesting finding was consistent with the result of Wang et al of which the number of viable cells derived from the same amount of frozen fat is small; the proliferation activity of such cells is good.³⁵ It is suggested that cells with better proliferation ability may be retained compare with poorer profile cells eliminated during cryopreservation. Further studies on stronger survival cell line to investigate biological or genetic characteristics overcoming extreme environments may be helpful to establish better cell preservation methods.

There are few limitations in our study. The exposure to oxygen, one of the main causes of LPO, was not controlled or measured. Furthermore, only one freezing and thawing protocol was used and CPA was not used. Which may be the cause of extensive cell necrosis occurred in first few days. Thus, considerably small portion of viable cell populations were taken into analysis, which may have increased bias to the result. However, these imprecisions were designed to represent clinical environment where left-over fat tissues are stored in the freezer in a syringe or plastic bags. And CPA was not considered, because its safety issue in human body is not clearly proven. However, further optimization of cryopreservation methods and technological advancement in safe CPA development may improve these results. The serial changes in LPO parameters could be observed for past 12 weeks, but it was resigned because viable cell was hard to find and gross tissue morphology collapsed into a brittle oily lump with low pH which was not suitable for clinical uses anyhow.

5 | CONCLUSION

Consistent with other studies, cell viability of the cryopreserved lipoaspirate in our study showed dramatic decrease in the first week and only small portion of the cell persist to proliferate until 12 weeks in the samples stored in -80°C only. The influence of LPO must be taken into consideration in fat tissues stored in 4°C and -20°C , as the by-product of LPO starts to increase after 2 weeks. Effective and safe CPA and antioxidant development in the future may partly solve these obstacles. Thus, until the adequate prevention protocol is developed, it is

not recommended to use fat tissue stored in 4°C for more than 2 weeks. Even for using the fat stored in -80°C , surgeon should keep in mind of the scantiness of viable and functional cells in the graft.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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