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Viability of Fat Cells in Frozen Fat Tissue in Relation to Thawing Technique

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Background: Damage of frozen fat, which will be used for retransplantation, is inevitable. Reuse of frozen fat requires a thawing process. No standardized method has yet been established for thawing frozen fat.
Methods: Microscopic analysis of count and viability of frozen fat of 21 patients. Two fat samples from each patient were harvested and frozen at –20°C in a common commercial refrigerator for different freezing durations. Thawing of fat samples was done. There was one (3 mL) sample for each thawing technique; technique A included natural thawing at 25°C for 15 minutes, while rapid thawing at 37°C for 10 minutes in a water bath was included in technique B. Survival rates of adipocytes were assessed with trypan blue staining. Culturing of adipose-derived stem cells to assess their ability to divide was done. Relating survival rate of frozen fat to patients' age and to duration of freezing was done. Results were statistically analyzed.
Results: The count of viable adipocytes is higher in technique A. Adipose-derived stem cells of frozen fat do not have the ability to divide in culture media. Viable adipocytes were higher in younger ages and in shorter freezing duration.
Conclusion: Natural thawing is better in maintaining frozen adipocyte viability. Younger patients will benefit from frozen fat more than older ones. Duration of freezing should not exceed 7 months. *(Plast Reconstr Surg Glob Open 2022;10:e4505; doi: 10.1097/GOX.0000000000004505; Published online 14 September 2022.)*

INTRODUCTION

Autogenous fat graft is a simple procedure that is used as soft tissue augmentation in the field of aesthetic and reconstructive plastic surgery, and it does not show foreign body or immune reactions and has abundant donor sites. Therefore, it is used widely in the scope of plastic and reconstructive surgery, and its clinical application is continuously expanding.[1] However, its main disadvantage is that it is difficult to predict the survival rates of transplanted fat due to absorption or necrosis of a variable portion of the transplanted adipose tissue after autogenous fat transfer.[2]

The viability of grafted fat has been reported to be very variable, in the range of 30%–80%, and is the subject of continuous debate.[3–8] The reasons for this variability include different donor site preparation techniques, harvesting methods, instruments used, fat grafting and injection techniques, grafting interval, and methods of analysis. All of these variations have made it difficult to compare studies.[9,10] Overcorrection or retransplantation has been implemented to address the high absorption rates of adipose tissue. However, since overcorrection leads to unnatural cosmetic outcomes and retransplantation brings with it the burden of resurgery, some recent studies aimed to freeze or cryopreserve residual adipose tissue after a fat transfer to be reused in case that retransplantation is needed.[11]

Significant amounts of adipose-derived stem cells (ADSCs) have been found in aspirated fat tissues.[12] These stem cells are known to differentiate into fat, cartilage, bone, muscle, and nerve. ADSCs have become a focus of attention in regenerative medicine.[13]

When frozen or cryopreserved fat is used after storage, the survival rate of the adipocytes is important since they have a great impact on the engraftment of adipose tissue after the reinjection.[11] Liu et al[14] reported that cells maintain some degree of metabolic activity because they are only partially frozen at –20°C, which is the typical storage temperature provided by commercial freezers. The glass-transition temperature of water that completely stops a

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cell's metabolic activity is –130°C. Therefore, many laboratories use –70°C as the appropriate temperature for their "deep freezer" to store cells and tissues.[15] Fat that has been stored for 3–12 months has been successfully used for regrafting. However, there is limited information on the clinical outcome of regrafting using stored fat tissues.[15]

Although a number of studies have examined the fat sampling and freezing processes,[16–18] relatively less analysis of the thawing process has been performed. Because the thawing process is essential for the reuse of the frozen autologous fat, appropriate thawing methods need to be studied to minimize cell damage.[19] Therefore, this study aimed to objectively analyze the difference in the survival rates of adipocytes depending on the thawing technique, and it also assessed the late decline of viability in fat cells, over time, for fat tissue stored at –20°C in a common commercial refrigerator that is used by many aesthetic surgeons.

Takeaways

Question: Is frozen fat effective in lipo-filling?
Findings: The count of viable adipocytes is higher in technique A. Adipose-derived stem cells of frozen fat do not have the ability to divide in culture media. Viable adipocytes were higher in younger ages and in shorter freezing duration.
Meaning: Natural thawing is better in maintaining frozen adipocyte viability.

MATERIALS AND METHODS

A total of 19 female patients and two male patients, who had no concomitant medical conditions, were included in the current study. The patients' age ranged between 28 and 57 years; the mean age of patients was 43.3 years ± 9.0, while the median age was 43 years. This study was approved by the research ethics committee. After obtaining approval from the patients and written consent to participate, patients were given an adequate explanation of the research on human-derived material, and they gave their consent before the experiment. The study was done in the period between May 2020 and September 2021.

Fat Harvest and Freezing

The area of fat harvest from the central infraumbilical area of the donor's abdomen was first infilterated with tumescent solution (500-mL saline and 1 ampule of 1-mg/mL epinephrine). After 20 minutes, the fat was harvested using a blunt-tipped suction cannula measuring 4.0 mm in diameter connected to a 60-mL wide-pore Ryle syringe. Lipoaspirate was harvested by one surgeon. The lipoaspirates (approximately 300–500 mL) were collected in multiple wide-pore Ryle syringes at the time of liposuction, and left in the standing position for at least 30 minutes; the lipoaspirates were then separated spontaneously into fat and fluids. The supernatant lipid layer and the bottom plasma and aqueous layer were removed, and only the middle layer, which contained the fat cells, was saved. Autologous fat transfer was carried out with these fats, while 6 mL of the residual adipose tissues per patient, which divided into two 3-mL samples in two 3-mL syringes, were placed directly into the commercial refrigerator freezer at –20°C to be used for microscopic analysis. The frozen samples were then retrieved and thawed. Samples were evaluated after different durations of freezing.

Thawing Techniques

The current methods of thawing frozen fats in clinical use were used and compared in this study. Two 3-mL samples per patient were examined, one 3-mL sample for each thawing technique. In thawing technique A, natural thawing at room temperature at 25°C for 15 minutes was used, while rapid thawing in a warm water bath at 37°C for 10 minutes was used in thawing technique B. The thawing time was set on the basis of the time needed for samples to reach the thawing temperature in each experimental technique in a preliminary study.[19]

Isolation, Counting, and Culturing of Adipose-derived Stem Cells

The used protocol was a modification of Lu et al.[20] The lipoaspirate was washed extensively (4–6 times) with phosphate buffer saline. The minced adipose tissue was collected in a Falcon tube 15 mL (Nalge Nunc International, Rochester, N.Y.). The minced fat was digested in the Falcon tube by 0.2% collagenase type I solution (Collagenase NB4 Standard; SERVA Electrophoresis, Heidelberg, Germany) at 37°C under constant shaking in a water bath shaker (Water Bath Incubator BT 25 Yamato Scientific, Tokyo, Japan) for 50 minutes. At the end of this procedure, the fat was completely digested and the solution became homogenous. The number of live adipocytes in each sample was counted by staining a 50 µL from the sample with 50 µL trypan blue dye (Sigma-Aldrich, St. Louis, Mo.), placed on a hemocytometer, and examined under an inverted microscope (200; Zeiss, Mount Vernon, Wash.); the dead cells will take on the stain, whereas the viable cells will not take on the stain.

The collagenase was neutralized by adding an equal volume of Dulbecco's Modified Eagle's medium (Lonza, Verviers, Belgium) with 13% fetal bovine serum (Lonza, Verviers, Belgium) to the solution. The cell suspension was centrifuged at 1300 rpm for 5 minutes. Cell pellet was formed at the bottom of the Falcon tube and that pellet was termed the stromal vascular fraction (SVF). The supernatant was carefully removed by pipette leaving the pelleted SVF.

The cell pellet was resuspended in a 10-mL complete culture medium formed of Dulbecco's Modified Eagle's medium, 13% fetal bovine serum, and 1.5% penicillin streptomycin mixture (Lonza, Verviers, Belgium). The number of adipocyte tissue stem cells (AT-ASCs) in each sample was counted also by the same technique.

The cell suspension was cultured in a culture flask 25 cm^2 (Easy Flask; Nalge Nunc International, Rochester, N.Y.) and incubated in a CO_2 incubator (NU 4950E, Autoflow Water Jacketed CO_2 incubato; NuAire, Plymouth, MN) at 37°C and 5% CO_2. The medium was replaced every

3 days, the nonadherent cells were discarded while the attached cells were washed with phosphate buffer saline, and the ASCs expansion was followed up by examination with inverted microscope (Axiovert 100, Zeiss-Germany). Cultured ASCs of passage were used without subculture.

We confirmed that ADSC expressed characteristic mesenchymal stem cell surface markers CD44 (91.8%) and CD90 (95.5%) by using flow cytometry analysis, while CD 34% absence was 7.1%, and CD 45% absence was 3.9%. Upon tissue culturing of ADSC, the fixed adherent ADSCs were stained with Giemsa stain, and examination under inverted microscopy was done to test for confluence.

Statistical Methods

Data were revised for completeness and consistency. Data entry was done on Microsoft Excel workbook. Quantitative data were summarized by mean and standard deviation, while qualitative data were summarized by frequencies and percentages. The program used for data analysis is IBM SPSS statistics for windows version 23 (IBM Corp., Armonk, N.Y.). Student *t* test, Pearson correlation coefficient, and multiple linear regression were used in the analysis of this study. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Among the live cells observed in the 1-µL samples in the hemocytometer, only the cells whose diameter was greater than 50 µm were counted (the average diameter of the fresh live cells was 12–15 µm; after 1 week, it enlarged to 16–17.5 µm; and after 14 days, it was 20–22 µm) (Fig. 1). It was found that 10–127 cells/µL (mean, 45.9 cells/µL) were counted in the samples naturally thawed at room temperature, while 3–54 cells/µL (mean, 18.1 cells/µL) were counted in the samples thawed rapidly. Results show that a higher mean number of viable fat cells were counted in technique A compared with technique B, and the difference is highly significant statistically. Results also show that the mean difference of the number of viable fat cells in the two techniques is 60%. Meaning that technique B reduces the fat cells by around 60% compared with technique A, as shown in Table 1. Figure 2 represents

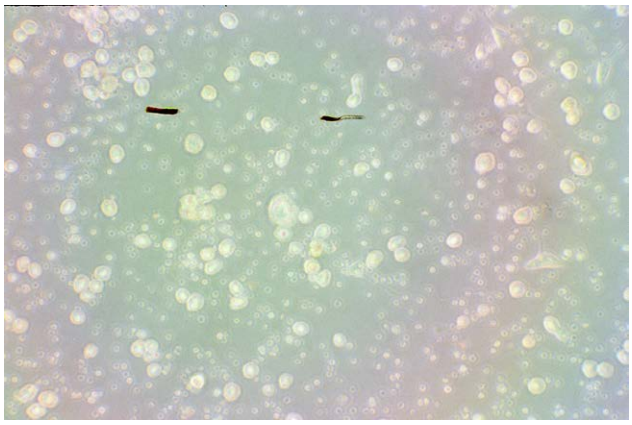

Fig. 1. This figure shows fat cells after thawing under inverted microscope (Axiovert 100, Zeiss-Germany), 200×.

Table 1. Comparison between Both Techniques as regards the Mean Number of Viable Fat Cells

Number of Viable Fat Cells	Technique A		Technique B			
	Mean	SD	Mean	SD	t	*P*
No. viable fat cells	**45.9**	28.7	18.1	11.6	4.1	0.000[a]

[a]$P < 0.01$ highly significant.

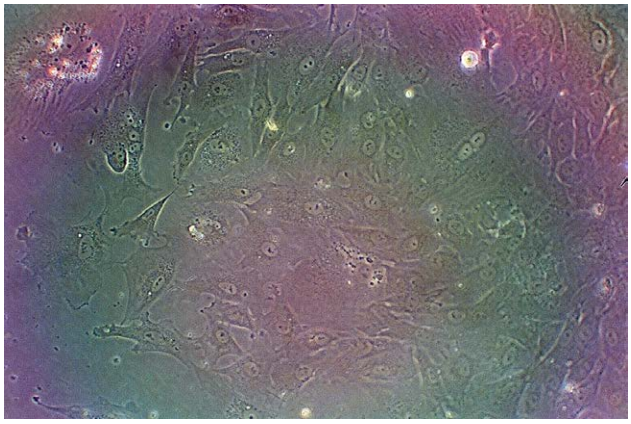

Fig. 2. This figure represents the fully confluent state of adipocyte tissue stem cells (AT-ASCs) in a fresh sample under inverted microscope (Axiovert 100, Zeiss-Germany), 200×.

the fully confluent state upon tissue culturing of ADSCs in a fresh sample under inverted microscope, while cultured ADSC of the frozen fat tissue showed no confluent appearance (Figs. 3, 4).

Correlation coefficient study between the age of patients and the number of viable fat cells in both thawing techniques showed a significant negative correlation between age of patients and the number of viable fat cells in the normal thawing technique (technique A), meaning that the higher the age, the lower the number of viable fat cells, as shown in Table 2 and Figure 5. Table 2 and Figure 5 also show a border line significant negative correlation between age of patients and the number of viable fat cells in the rapid thawing technique (technique B).

Correlation coefficient study between the duration of freezing (freezing interval) and the number of viable fat cells in both techniques showed a highly significant negative correlation between duration of freezing and the number of viable fat cells in thawing technique A, as shown in Table 3 and Figures. 6, 7. Table 3 and Figures 6, 7 also show a highly significant negative correlation between duration of freezing and the number of viable fat cells in thawing technique B.

Comparison between the mean number of viable fat cells in each technique and the duration of freezing showed a higher mean number of viable fat cells in technique A among those samples frozen for less than 8 months duration with a mean number of 68.7, compared with those frozen for more than 8 months duration with a mean number of 25.2, and the difference was highly significant statistically. The mean difference was lower by 63% when frozen more than 8 months duration. Results

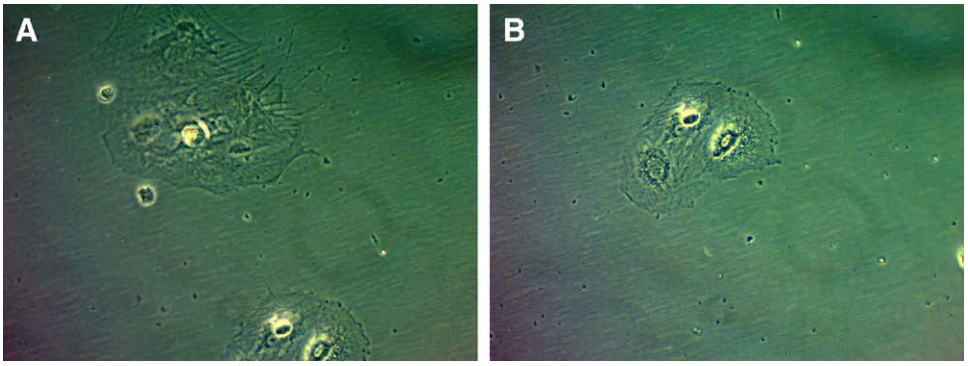

Fig. 3. A and B, Poor proliferation of adipocyte tissue stem cells (AT-ASCs) upon culturing of frozen fat thawed at room temperature for 15 minutes (technique A), showing formation of large cells (hungry cells) under inverted microscope (Axiovert 100, Zeiss-Germany), 200×.

also showed a higher mean number of viable fat cells in technique B among those samples frozen for less than 8 months duration with a mean number of 26.5 compared with those frozen for more than 8 months duration with a mean number of 10.5, and the difference was also highly significant statistically. The mean difference was lower by 60% when frozen for more than 8 months duration, as shown in Table 4.

DISCUSSION

Because the thawing process is essential for the reuse of frozen autologous fat, appropriate thawing methods need to be studied to minimize cell damage. Few studies for the analysis of thawing process of frozen fat have been performed. To find a better thawing method among those commonly used in clinic, previous studies were performed, such as Pu et al,[21] who suggested that outcomes were better when cryopreserved fat was thawed at 37°C. A study on the survival rates of adipocytes is normally performed by many techniques, such as live cell counting through trypan blue staining,[21,22] the measurement of enzyme activity using glycerol-3-phosphate dehydrogenase analysis,[21,23] the measurement of mitochondrial activity using XTT assay[2,17] or MTT assay,[18,24] and the observation of cell morphology through hematoxylin and eosin staining.[21]

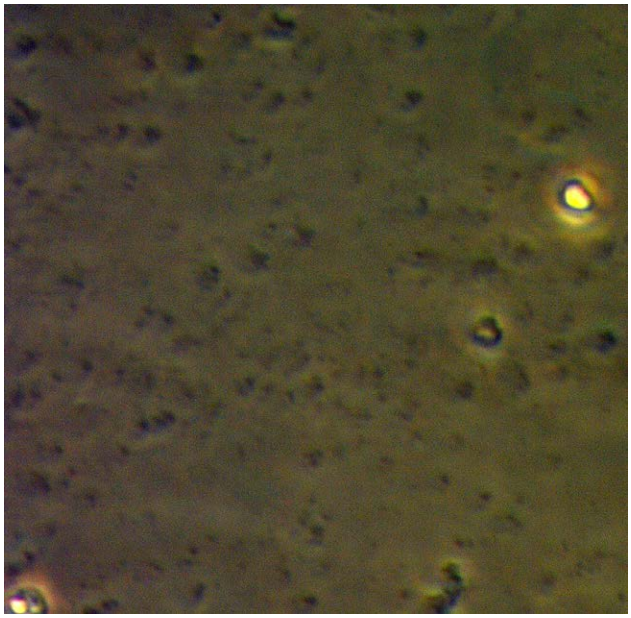

Fig. 4. This figure shows no proliferation or adhesion of adipocyte tissue stem cells (AT-ASCs) upon culturing of frozen fat rapidly thawed at 37°C in water bath for 10 minutes (technique B) under inverted microscope (Axiovert 100, Zeiss-Germany), 200×.

The survival rates of adipocytes were assessed by measuring the volume of the fat layer in the top layers separated after centrifugation, counting the number of live adipocytes after staining with trypan blue, and measuring the activity of mitochondria in the adipocytes in the previous study by Hwang et al.[19] Eto et al[25] observed that living and dead adipocytes can be differentiated not only with hematoxylin and eosin staining but also with immunohistochemistry for perilipin. In the current study, the survival rate was assessed by counting the number of viable adipocytes after staining with trypan blue, and then stem cell isolation, and culturing of ADSC of frozen fat tissue.

In the current study, it was found that 10–127 cells/μL (mean, 45.9 cells/μL) were counted in the samples naturally thawed at room temperature, while 3–54 cells/μL (mean, 18.1 cells/μL) were counted in the samples thawed rapidly (Table 1). Our results show that a higher mean number of viable fat cells were counted in thawing technique A compared with thawing technique B, and the difference was highly significant statistically. Our results are contrary to previous results by Hwang et al,[19] who found that 3–37 cells/μL (mean, 19.2 cells/μL) were counted in the group naturally thawed at room temperature, and

Table 2. Correlation Coefficient between the Age of Patients and the Number of Viable Fat Cells in Both Techniques

Number of Viable Fat Cells	Age
No. viable fat cells in technique A	r = –0.551 *P = 0.01
No. viable fat cells in technique B	r=-0.410 P = 0.06

*P < 0.05 significant

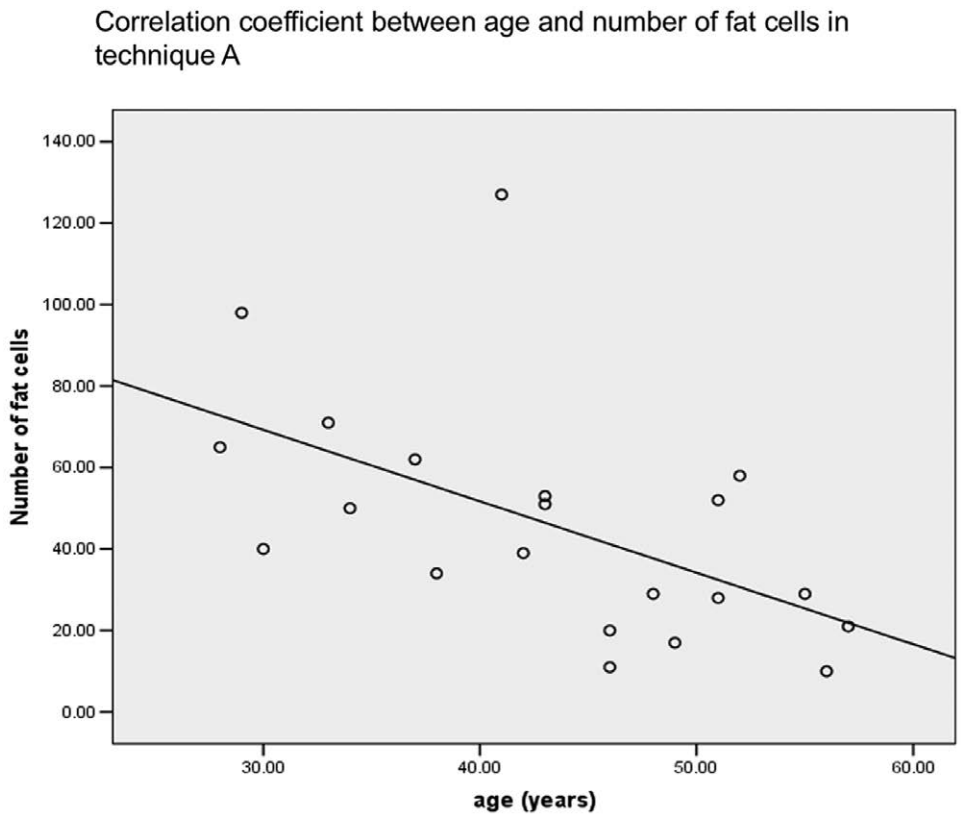

Fig. 5. Correlation coefficient between age of patients and the number of viable fat cells in technique A (Table 2).

8–60 cells/μL (mean, 27.9 cells/μL) in the group thawed rapidly.

Results of the Hwang et al[19] study showed that in the group with rapid thawing for 10 minutes in a water bath, it was observed that the cell count of live adipocytes was significantly higher. This showed a similar pattern to that observed in experiments involving the thawing of other cryopreserved human tissues or cells, such as sperm in a study by Martinez-Soto et al[26] or fibroblasts, in which the experimental group that was thawed rapidly at 37°C showed higher cell viability.[27–29] However, this was in contrast to our study results in which we observed that natural thawing of frozen fat at room temperature will keep a higher number of viable cells and minimize adipocyte damage during thawing, and accordingly considered that injection of fat that has undergone natural thawing at room temperature should yield better outcomes.

The degree of adipocyte damage during the harvesting and storage process will be variable in each sample. In addition, the variability in adipocyte viability according to the age of the person whose fat was sampled should be taken into account, as should the duration of freezing. In the current study when correlating the age of patients and duration of freezing to the survival rate of frozen fat cells, we found a significant negative correlation between the age of patients, the duration of freezing, and the survival rate of the frozen fat cells.

Previous studies on the time-dependant viability of cryopreserved fat tissues have shown a wide range of results. Schuller-Petrovic[30] reported that slow freezing of the tissue to –20°C, shortly after harvesting had no harmful effect on the adipocytes. Sommer and Sattler[22] reported that live adipocytes were found after cryopreservation at –20°C for 3 years. However, Wolter et al[31] reported that the adipocytes were destroyed after 48 hours of freezing at –20°C and that reuse of adipose tissues cryopreserved at –20°C provides an injection of mostly dead cells. These contradicting results have led to confusion about the effect of cryopreservation on adipose tissues. Results of the current study showed a higher mean number of viable fat cells in both thawing techniques among those samples frozen for less than 8 months duration, compared with those frozen for more than 8 months duration, and the difference was highly significant statistically.

Table 3. Correlation Coefficient between the Duration of Freezing and the Number of Viable Fat Cells in Both Techniques

Number of Viable Fat Cells	Duration of Freezing
No. viable fat cells in technique A	R = –0.909 *P = 0.000
No. viable fat cells in technique B	R = –0.845 *P = 0.000

*P < 0.01 highly significant.

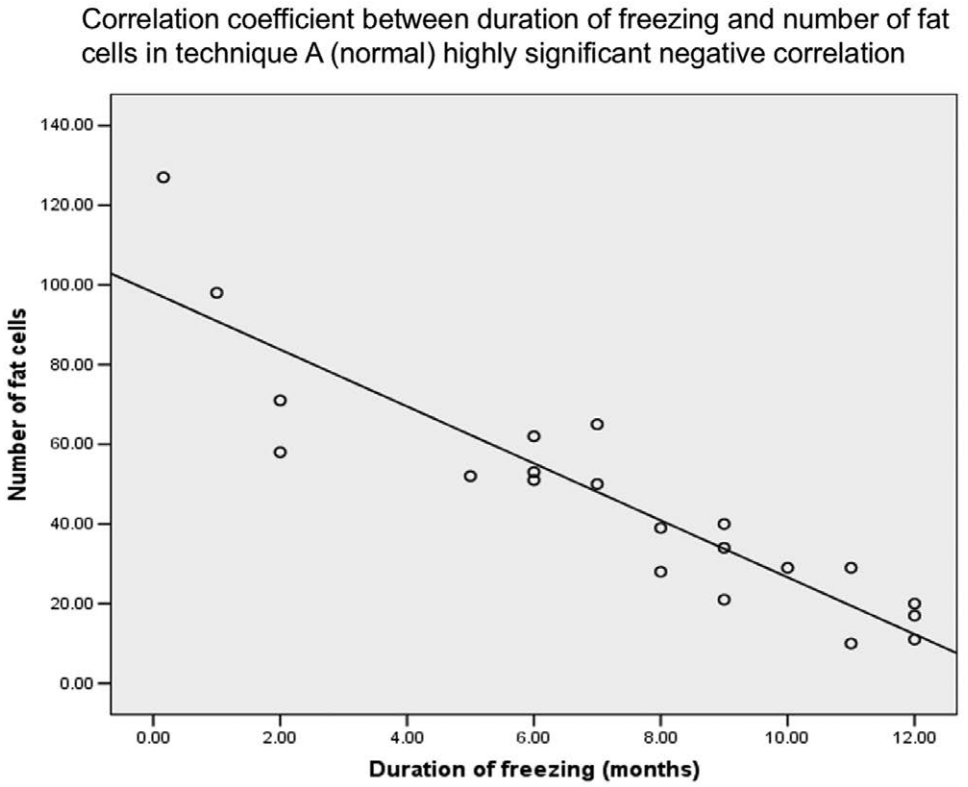

Fig. 6. Correlation coefficient between duration of freezing and the number of viable fat cells in technique A (normal) highly significant negative correlation (Table 3).

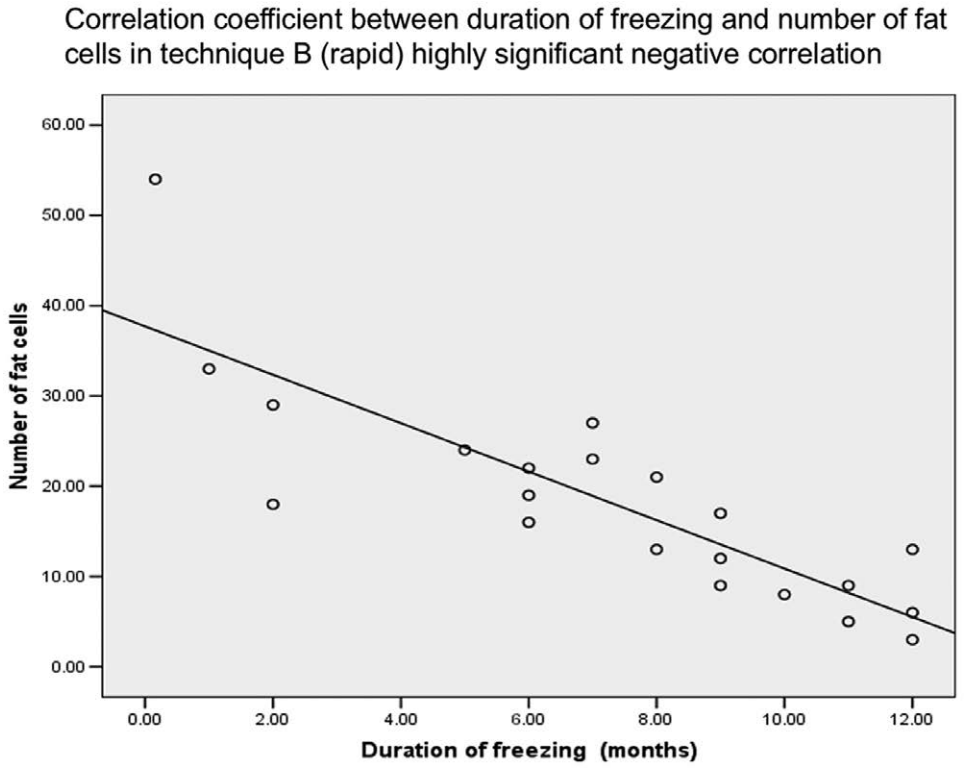

Fig. 7. Correlation coefficient between duration of freezing and the number of viable fat cells in technique B (rapid) highly significant negative correlation (Table 3).

Table 4. Comparison between the Number of Viable Fat Cells in Each Technique and Duration of Freezing

	<8 Mo Freezing Interval, N = 10		≥8 Mo Freezing Interval, N = 11			
N = 21	Mean	SD	Mean	SD	t	*P*
No. viable fat cells in technique A	68.7	24.9	25.2	10.3	5.2	0.000*
No. viable fat cells in technique B	26.5	10.9	10.5	5.3	4.3	0.000*

*$P < 0.01$ highly significant.

Trypan blue staining or XTT assay has a limitation that the results come from not only adipocytes but also other live cells.[19] Therefore, to build on these findings, further studies should be performed by harvesting each sample under more uniform conditions, including a more homogeneous patient sample and more standardized procedures. Furthermore, to more thoroughly understand the changes in adipocytes depending on the thawing methods, not only in vitro studies on adipocytes but also more advanced studies on the difference in the survival rates of adipocytes after the actual in vivo transplantation are needed. The study included a small number of samples; in the future, a study with a larger number of individuals would be beneficial. Although this study aimed to compare frozen fat and compare the thawing techniques, it may be useful to compare these numbers to fresh unfrozen fat cells.

CONCLUSIONS

The current study concluded and recommended that, the higher the age, the lower the mean number of viable fat cells in frozen fat transfer. The duration of freezing should not exceed 7 months, as the mean number of viable fat cells is affected by the duration of freezing (8 months is the median time of freezing in the current study). Technique A, or natural thawing at room temperature, will lead to much higher viable fat cells compared with rapid thawing at 37° for 10 minutes in technique B, as proved by the higher mean number of viable fat cells thawed by this technique.

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