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EFFICIENT PROCEDURE FOR HUMAN ADIPOSE TISSUE CRYOPRESERVATION WITHOUT SPECIALIZED FREEZING EQUIPMENT

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Abstract

BACKGROUND: Adipose tissue grafting is one of the reconstruction methods for damaged tissue repair. **OBJECTIVE:** To develop a convenient procedure for human adipose tissue cryopreservation without any special equipment. **MATERIALS AND METHODS:** Adipose tissues were frozen using different combinations of permeating and non-permeating cryoprotectants at a cooling rate of -1°C/min and stored at -20°C for 1, 3, 6 and 9 months. Histo-morphological characteristics, mitochondrial activity, oil ratio (OR) index, survival and differentiation potential of mesenchymal stem cells of thawed adipose tissue were evaluated. **RESULTS:** The most damage or degeneration and OR indices of adipose tissues were detected in phosphate-buffered saline without any cryoprotectant at 1, 3, 6, and 9 months after cryopreservation ($P \leq 0.05$). The best protection of adipose tissue against freezing damage was observed when using a solution of 0.5 M DMSO + 9% FBS + 0.2 M trehalose ($P \leq 0.05$). Similarly, mitochondrial activities of thawed adipose tissues were the highest in the 0.5 M DMSO + 9% FBS + 0.2 M trehalose, but lowest in the phosphate-buffered saline. There was no difference in the stemness and differentiation potential of adipose tissue-derived mesenchymal stem cells among different cryopreservation treatments. **CONCLUSION:** The combination of 0.5 M DMSO, 9% FBS and 0.2 M trehalose has the best protection for human adipose tissue during cryopreservation.

Keywords: adipose tissue; cryoprotectant; freezing.

INTRODUCTION

Adipose tissue grafting is a cosmetic and reconstructive procedure for patients with lipodystrophy or contour deformities caused by disease, trauma, congenital and acquired defects, tumor removal, or for patients interested in skin rejuvenation (1). Adipose tissue has advantages due to its availability, low cost, easy processing, good biocompatibility and tissue integration, no allergic/immunological reactions and minimal patient's discomfort (2). The extra adipose tissue

extracted by liposuction may be discarded if not used up in filling or stored at -20°C for future use. Cryopreservation of such adipose tissues for subsequent applications not only saves costs, but also reduces patient discomfort and possible complications for repeated liposuctions (3). Lipoatrophy after lipofilling increases the need for frequent liposuction in patients to obtain extra adipose tissues for further correction and re-injection (4, 5).

Adipose tissue cryopreservation needs to protect both the stromal vascular fraction (SVF) and the integral extracellular matrix (ECM). SVF is composed of a heterogeneous population of different cells, including adipocytes, fibroblasts, adipocyte progenitor cells, vascular endothelial cells, smooth muscle cells, endothelial cells, hematopoietic cells, and mesenchymal cells (6, 7). The preservation of both SVF and ECM reduces tissue calcification, sebaceous oil cysts, granuloma, hematoma, necrosis, inflammation, and ultimately lipoatrophy after transplantation (8, 9, 10, 11). There are two kinds of cryoprotectants, permeating and non-permeating. Permeating CPAs interact with water through hydrogen bonding, decreasing the freezing point of water and reducing damage caused by intracellular ice crystals (12). Non-permeating CPAs remain in the extracellular space and exert their protective effect by reducing osmotic stress and reducing the formation of ice crystals (5, 13, 14, 15, 16, 17, 18, 19, 20). Often two or more CPAs are combined to reduce the concentrations of individual CPAs and their toxicity. Adipose tissue requires various CPAs to effectively preserve both ECM and cells of multiple types.

This study aimed to develop a method for efficient adipose tissue cryopreservation for patients with leftover adipose tissues for use in clinics without special equipment such as nitrogen tanks or -80°C freezer. Different combinations of CPAs were evaluated for their protective effects on patient's adipose tissues at -20°C.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Motamed Academic Research Institute (IR.ACECR.IBCRC.REC.1400.012). Except where indicated, chemicals were from Sigma-Aldrich Co. (St. Louis, MO, USA).

Experimental design

Human adipose tissues were obtained from healthy donors with the informed consent in writing. Donors were 23 to 57 years old. Drug addiction, and those with metabolic diseases, liver disorders, bacterial infection, mycoplasma infection and viral infections (HIV, HBV, HCV and HPLV) were excluded. Adipose tissues were frozen in five experimental treatments (negative control, NCG; conventional freezing medium, CFM; first intervention, FI; second intervention, SI; third intervention, TI) with fresh adipose tissues as control, and stored for 1, 3, 6, and 9 months at the -20°C freezer.

Adipose tissue cryopreservation

The supernatant (oil) and bottom aqueous plasma fraction were discarded. The middle fat layer was collected and washed three times with phosphate-buffered saline containing antibiotics (penicillin, streptomycin and amphotericin B). Prepared adipose tissues were equally divided into six experimental groups: positive control group (fresh adipose tissues), negative control group (NCG, PBS without CPA), conventional freezing medium (CFM, 0.2 M DMSO and 25% sucrose), first intervention (FI, 0.2 M DMSO, 25% sucrose, and 9% FBS), second intervention (SI, 0.5 M DMSO and 0.2 M trehalose), and third intervention (TI, 0.5 M DMSO, 9% FBS, and 0.2 M trehalose). In all experiments, 1-mL CPA solution was added to 1-mL adipose tissue. The mixture in cryovials were cooled in a controlled-rate freezer at -1 °C/min to -20°C for storage.

For thawing, cryovials were removed from the freezer and immediately placed in a 37°C water bath for 1~2 min. Thawed samples were evaluated for morphological characteristics, mitochondrial activity and oil ratio index. Mesenchymal stem cell density, survival and differentiation potential were also compared to the fresh tissue samples.

Histomorphological evaluation

Adipose samples were fixed in 10% formalin solution and routinely processed. Tissue slices (5 μm) were made using a microtome and stained with hematoxylin and eosin (H&E). Slides were examined using a light microscope (BX 53, Olympus Corp., Tokyo, Japan) at 10x and 40x magnifications. Tissue necrosis or degeneration, calcification, cyst or any other abnormal mass, and morphological characteristics of adipocytes were determined. Two pathologists evaluated all prepared slides.

Oil ratio estimation

Oil ratio (OR) is a specific index to estimate adipose damage or degeneration. OR indices after cryopreservation were compared to the fresh adipose tissues. In order to calculate OR, adipose tissue was centrifuged at 2300×g for 5 min. Oil and fat volume were determined for the OR calculation by

$$\mathrm{OR}=\frac{\text{Oil volume}}{\text{Oil volume}+\text{Fat volume}}$$

Cell survival and mitochondrial activity

MTT assay was used to determine viability and mitochondrial oxidoreductase activity of fresh and thawed adipose tissues. Approximately 1 mL of adipose tissue was incubated with 1 mL of MTT at 250 mg/mL for 3 h. After incubation, the tissue was transferred to 1 mL of DMSO/methanol (50:50) and incubated for 1, 6, 12, and 24 h. Two-hundred mL of the eluate was transferred to a 96-well plate, and optical density (OD) taken at 595 nm by a plate reader (Molecular Devices, Wokingham, UK). Explants were removed and left to dry for 24 h to increase accuracy before weighing. Tissue samples were dried overnight and then weighed. The survival index was calculated using the following formula:

$$Viability\ Index\ (VI)=\frac{\text{Average OD reading}}{\text{Dried tissue weight}}$$

$$Metabolic\ activity=\frac{\text{VI at each time}}{\text{VI of fresh sample}}\times 100$$

Adipose mesenchymal stem cells

Mesenchymal stem cells (MSCs) were isolated via enzymatic digestion as previously stated (21). Briefly, lipoaspirate tissues were digested with an equal volume of 0.01% type I collagenase at 37°C for 30 min. After PBS washing, SVF was filtered through a 150 µm nylon mesh and harvested by Ficoll separation to obtain the mononuclear cell fraction. Isolated cells were suspended in the DMEM medium containing 10% FBS (fetal bovine serum, Gibco, Germany), 1% NEaa, $NaHCO_3$ (3.7 mg/mL), L-glutamin and penicillin/streptomycin (100U/mL and 100 mg/mL, respectively) and incubated in 5% CO_2 and 37°C. Cultured cells were trypsinized using 0.05% trypsin/1 mM EDTA (Invitrogen, Germany) and cultured until passage 4. The total concentration of AD-MSCs in passage 4 was counted with a hemocytometer in all experimental and positive control groups.

To evaluate the AD-MSC quality, the expression of biomarkers (CD90, CD73, CD105, CD34, and CD45) was detected. The multiple differentiation potential into osteocytes, adipocytes and chondrocytes was also measured. For osteogenic differentiation, AD-MSCs at passage 4 were cultured for 28 d with differentiation medium composed of DMEM supplemented with 10% FCS, 50 µg/mL ascorbic 2-phosphate, 10 nM dexamethasone, and 10 mM β-glycerol phosphate. For chondrogenic differentiation, the proliferation medium was replaced with chondrogenic medium consisting of high-glucose DMEM supplemented with 0.1 µM dexamethasone, 10 ng/mL TGF-β1, 50 µg/mL ascorbic acid, and 50 mg/mL ITS+ premix (Becton Dickinson), 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 ng/mL selenium acid, and 10% FCS. For adipogenic differentiation, AD-MSCs were exposed to a medium that consisted of DMEM supplemented with 10% FCS, 50 µg/mL ascorbic 2-phosphate, 100 nM dexamethasone, and 50 µg/mL indomethacin. Differentiated cells (osteocyte, adipocyte and chondrocyte) were stained with 2% Alizarin Red S, Oil Red O, and toluidine blue solutions, respectively.

In addition to the differentiation potential of AD-MSCs, the qualitative validation of cells was also determined by flow cytometry analysis. For this purpose, MSCs in different experimental groups were incubated with a blocking solution of 3% serum in PBS for 30 min. After centrifugation, 5×10^5 cells were suspended in the blocking solution and stained with antibodies against human CD90, CD105, CD34, CD45, and CD73 (BD Biosciences). After incubation for 30 min, the expression of molecular markers was detected by flowcytometry (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Data were expressed as mean ± SE. Comparison between mean values was made by one-way ANOVA (SigmaStat, ver. 2).

RESULTS

Adipose morphology and OR indices

Histomorphology of fresh and thawed adipose tissues are shown in Figure 1. The type and severity of damage in frozen adipose tissues without any CPAs (NCG) were higher than the tissues that were frozen with CPAs ($P\leq0.05$).

Among adipose tissues frozen with CPAs (CFM, FI, SI, and TI), the highest tissue damages (diffuse tissue degeneration, numerous cysts, inflammatory cell infiltration, cell degeneration, etc.) were detected in the conventional freeze group (CFM) at all time points ($P \leq 0.05$). The best protection (the least tissue wrinkling, oil cysts, degeneration, and infiltration of inflammatory cells) were detected in the SI group (0.5 M DMSO + 0.2 M trehalose) and the TI group (0.5 M DMSO + 9% FBS + 0.2 M trehalose). There was no significant difference between the SI and TI groups ($P \geq 0.05$). Storage duration increased the damage as examined by microscopy ($P < 0.05$).

OR indices of adipose tissues cryopreserved for 1-, 3-, 6- and 9-months are shown in Table 1. OR of the fresh adipose was 5.10 ± 0.06. The highest OR indices were detected in the conventional freeze group (CFM) ($P \leq 0.05$). The best protection and the lowest OR indices were seen in the TI group (0.5 M DMSO + 9% FBS + 0.2 M trehalose) ($P \leq 0.05$), followed by the SI group (0.5 M DMSO + 0.2 M trehalose) ($P \leq 0.05$).

Mitochondrial activities of adipose tissues

The highest mitochondrial activity was detected in the fresh adipose tissue. After cryopreservation, the highest and lowest mitochondrial activities were detected in the TI group (0.5 M DMSO + 9% FBS + 0.2 M trehalose) and the NCG group (PBS without CPA), respectively (Table 2). The best time to evaluate the mitochondrial activities was up to

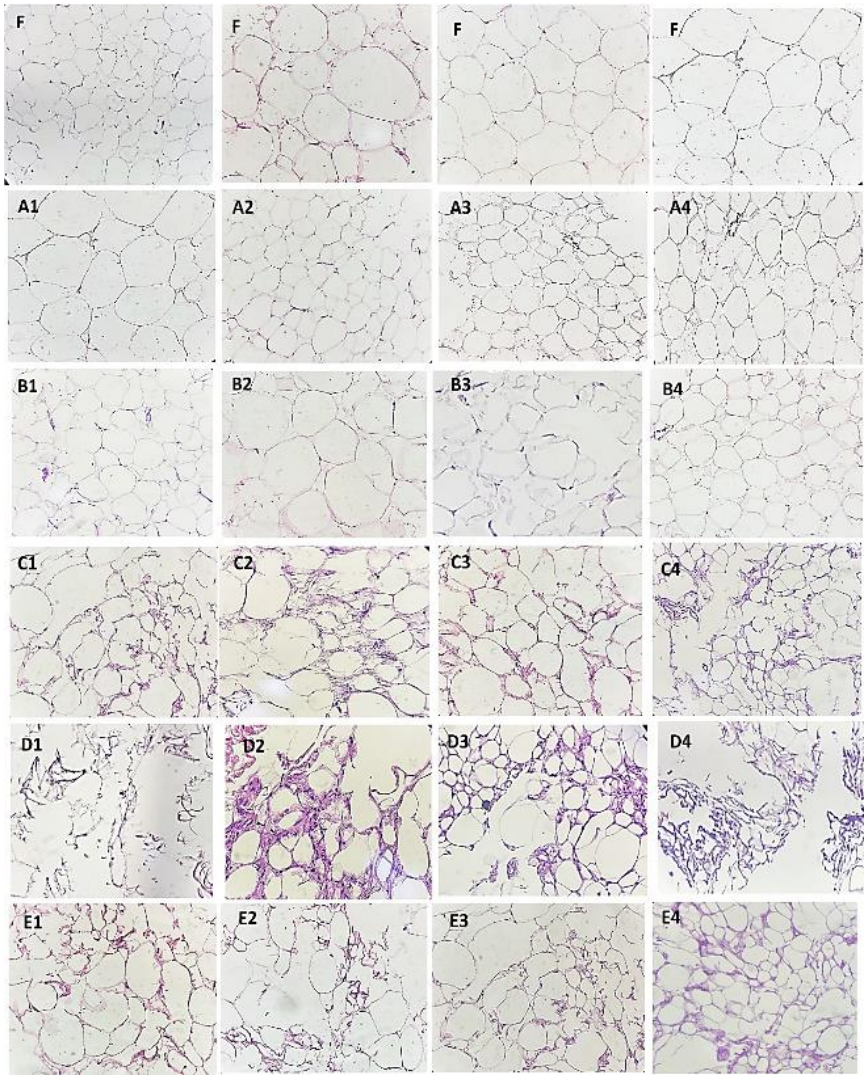

Figure 1. Histomorphological characteristics of fresh adipose tissue (F) and adipose tissues frozen in different cryoprotectant formulations (TI, A1-4; SI, B1-4; FI, C1-4; NCG, D1-4; CFM, E1-4) after 1 (A1, B1, C1, D1), 3 (A2, B2, C2, D2), 6 (A3, B3, C3, D3), and 9 (A4, B4, C4, D4) months. See the detailed description in the text.

24 h after the addition of DMSO/methanol using the VersaMax plate reader (Table 2).

Evaluations of AD-MSCs

Cell count and morphological feature of AD-MSCs are shown in Table 3 and Figure 2. Total cell densities in fresh adipose tissue were from 4.5~4.6 × 10^6 cells per 10 g adipose tissue ($P \leq 0.05$). There was no significant difference in cell densities between the fresh group and the TI group (0.5 M DMSO + 9% FBS + 0.2 M trehalose) after 1- and 3-month frozen storage. The most and least cell densities were detected in the TI group (0.5 M DMSO + 9% FBS + 0.2 M trehalose) and NCG group (PBS without CPA), respectively (Table 2). No significant difference was observed in the viability of mesenchymal stem cells derived from fresh and frozen-thawed adipose tissues ($P>0.05$).

Table 1. Oil ratio (OR) indices in the fresh and frozen-thawed adipose tissues. Data are presented as mean ± SE.

Groups /months	Fresh	Experimental groups (see the detailed description in the text)				
		NCG	CFM	TI	SI	FI
9	5.1±0.06^{a,A}	10.4±0.05^{b,A,B}	9.9±0.06^{c,A}	5.9±0.06^{d,A,B}	6.4±0.06^{e,A}	9.8±0.06^{c,A}
6	5.1±0.06^{a,A}	10.5±0.05^{b,B}	9.5±0.05^{c,B}	6±0.07^{d,e,A}	6.1±0.06^{d,B}	9.6±0.06^{c,A,B}
3	5.1±0.06^{a,A}	10.2±0.05^{b,B}	9.7±0.05^{c,A}	5.8±0.06^{d,A,B}	5.7±0.05^{d,C}	9.7±0.06^{c,A}
1	5.1±0.06^{a,A}	9.9±0.05^{b,C}	9.4±0.05^{c,B}	5.7±0.05^{d,B}	5.7±0.06^{d,C}	9.4±0.06^{c,B}

$^{A-E}$ Different superscript uppercase letters in the same column denote significant difference at p = 0.05).
$^{a-d}$ Different superscript lowercase letters in the same row denote significant difference at p = 0.05.

Table 2. Mitochondrial activities of adipose tissues. Data are presented as mean ± SE.

Months	Hrs	Treatment groups (see the detailed description in the text)					
		CFM	NCG	FI	SI	TI	Fresh
1	1	1.47±1.3^{a,A}	1.95±1.1^{b,A}	1.99±1.2^{b,A}	2.38±1.4^{c,A,D}	2.57±1.4^{c,A}	3.13±0.08^{d}
	12	1.51±0.9^{a,A}	1.72±1.02^{a,}	1.89±0.8^{b,c,A}	2.17±0.9^{c,A}	2.53±0.8^{d,A}	3.13±0.08^{e}
	24	1.48±1.4^{a,A}	1.98±1.9^{b,A}	1.97±1.2^{b,A}	2.58±1.3^{c,A,C,D}	2.51±1.4^{c,A}	3.13±0.08^{d}
	48	0.49±1.4^{a,B}	0.32±1.3^{a,B}	1.00±1.2^{b,B}	1.45±1.3^{c,B}	1.71±1.4^{c,B}	3.13±0.08^{d}
3	1	2.46±1.3^{a,b,C}	2.22±1.3^{a,C}	2.69±1.5^{b,c,C}	2.78±1.5^{b,c,d,C}	2.96±1.4^{c,d,C}	3.13±0.08^{d}
	12	2.58±1.5^{a,b,C}	2.31±1.3^{a,C}	2.27±1.25^{a,D}	2.76±1.5^{b,C}	2.77±1.6^{bC}	3.13±0.08^{c}
	24	2.60±1.4^{a,c,C}	2.18±1.3^{b,C}	2.3±1.3^{a,b,D}	2.49±1.5^{a,b,c,A,}	2.65±1.4^{c,C}	3.13±0.08^{d}
	48	1.44±1.5^{a,A}	1.39±1.4^{a,D}	1.50±1.4^{a,E}	1.43±1.6^{a,B}	1.39±1.5^{a,D}	3.13±0.08^{b}
6	1	2.97±1.4^{a,D}	2.11±0.9^{b,C}	2.64±1.4^{a,C}	2.63±1.4^{a,C,D}	2.96±1.2^{a,C}	3.13±0.08^{c}
	12	2.66±1.3^{a,C,D}	2.40±1.2^{a,b,}	2.24±1.6^{b,D}	2.48±1.6^{a,b,A,C}	2.97±1.4^{a,c,C}	3.13±0.08^{c}
	24	2.46±1.2^{a,C}	2.41±1.2^{a,C}	2.26±1.6^{a,D}	2.46±1.6^{a,C}	3.00±1.5^{b,C}	3.13±0.08^{b}
	48	0.86±0.6^{a,E}	1.13±0.7^{a,b,}	1.48±0.97^{b,E}	1.34±1.6^{b,B}	1.85±1.8^{c,B}	3.13±0.08^{d}
9	1	2.40±1.3^{a,b,C}	2.13±1.1^{a,C}	2.35±0.56^{a,b,}	2.53±1.5^{b,A,C}	2.91±1.5^{c,C}	3.13±0.08^{c}
	12	2.40±1.8^{a,C}	2.19±1.5^{a,b,}	2.02±1.5^{b,D}	2.23±1.6^{a,b,A}	2.22±1.6^{a,b,A}	3.13±0.08^{c}
	24	2.49±1.2^{a,C}	2.10±1.3^{b,C}	2.04±1.5^{b,D}	2.27±1.6^{b,A}	2.38±1.5^{b,A}	3.13±0.08^{c}
	48	1.53±1.2^{a,A}	1.85±1.1^{b,A}	1.99±1.5^{b,A}	1.28±1.5^{a,B}	1.38±1.5^{a,D}	3.13±0.08^{C}

$^{A-E}$ Different superscript uppercase letters in the same column denote significant difference at p = 0.05). $^{a-d}$ Different superscript lowercase letters in the same row denote significant difference at p = 0.05.

Cultured mesenchymal cells in all groups are shown on days 48, 72, 96, 120 and 144 h after cultivation (Figure 2).

Differentiation potential of mesenchymal stem cells into chondrocytes, osteocytes, and adipocytes is demonstrated in Figure 3. There was no significant difference (P>0.05) in the differentiation potential between fresh and cryopreserved groups. In addition, there was no significant difference in the expression of CD73, CD105, CD90, CD34, and CD45 markers on MSCs derived from different treatment groups. The expression of CD90, CD73 and CD105 in MSCs was 99.1%, 99.8% and 97.8%, respectively. MSCs did not express CD34 and CD45 (with 99.6% and 99.3% accuracy, Figure 4). Due to the large number of graphs in the different control and experimental groups, only

Table 3. Total cell count (×10^6) of MSCs-derived adipose tissue in the fresh and frozen-thawed adipose tissues. Data are presented as mean ± SE.

Months	Experimental groups (see the detailed description in text)					
	FI	SI	TI	CFM	NCG	Fresh
1	$3.213\pm0.002^{a,A}$	$4.253\pm0.005^{b,A}$	$4.528\pm0.001^{c,A}$	$2.837\pm0.001^{d,A}$	$2.608\pm0.001^{d,A}$	$4.604\pm0.003^{c,A}$
3	$3.103\pm0.004^{a,A}$	$4.092\pm0.005^{b,B}$	$4.578\pm0.003^{c,B}$	$2.432\pm0.001^{d,B}$	$2.124\pm0.002^{e,B}$	$4.581\pm0.003^{c,A}$
6	$2.311\pm0.002^{a,B}$	$3.360\pm0.009^{b,C}$	$3.520\pm0.002^{c,C}$	$1.608\pm0.001^{d,C}$	$1.218\pm0.001^{e,C}$	$4.590\pm0.002^{f,A}$
9	$1.896\pm0.001^{a,C}$	$2.410\pm0.003^{b,D}$	$3.498\pm0.003^{c,C}$	$1.595\pm0.001^{d,C}$	$1.153\pm0.001^{e,D}$	$4.575\pm0.003^{f,A}$

$^{A-E}$ Different superscript uppercase letters in the same column denote significant difference at p = 0.05). $^{a-d}$ Different superscript lowercase letters in the same row denote significant difference at p = 0.05.

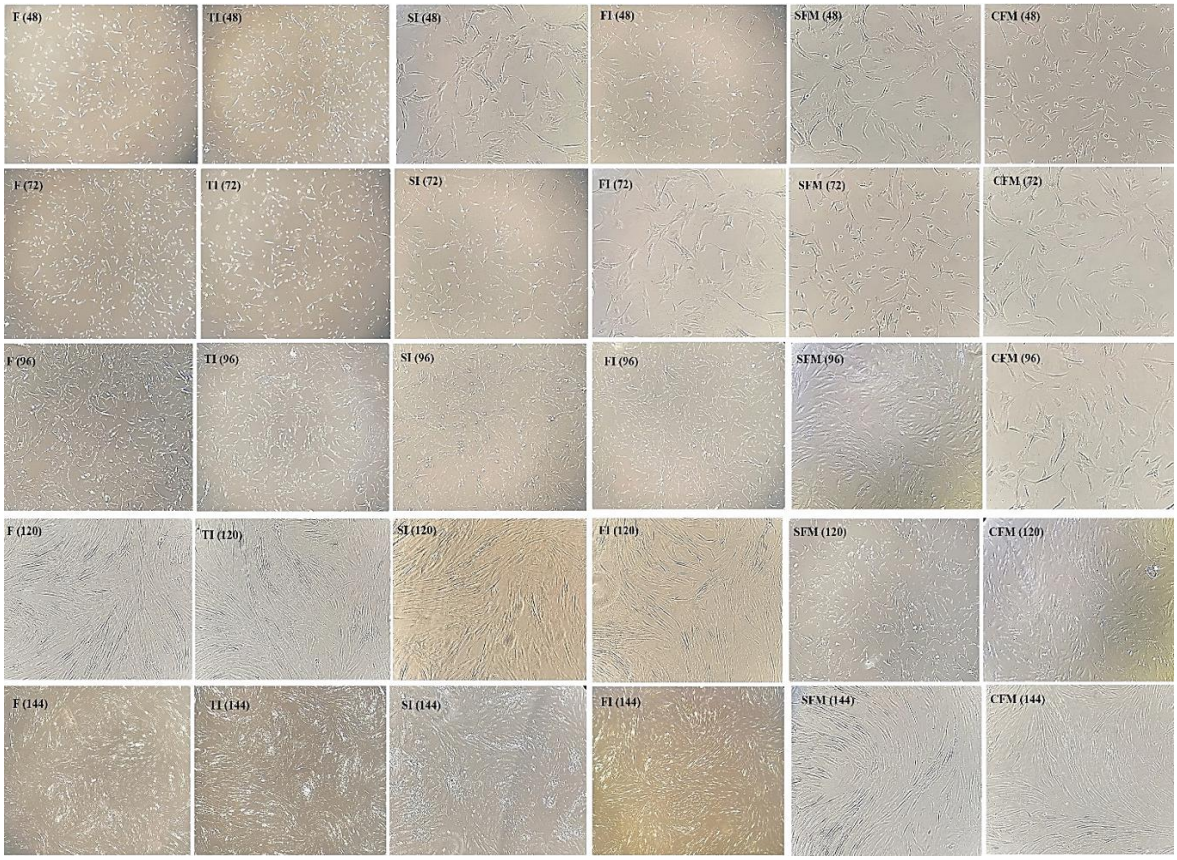

Figure 2. Adipose-derived mesenchymal stem cells from fresh tissues (F), negative control (NCG), conventional freezing medium (CFM), first intervention (FI), second intervention (SI), and third intervention (TI) after 48, 72, 96, 120 and 144 h. See the detailed description in the text.

the expression of CD73, CD105, CD90, CD34, and CD45 markers in the TI group are shown in Figure 4.

DISCUSSION

Efficient cryopreservation of adipose tissues can facilitate adipose tissue grafting and MSC therapies (22). Cryopreservation of adipose tissue is still a big challenge, because of the different cell types and the complex ECM network in adipose tissue (12, 23).

We have shown that the best morphological characteristics of frozen-thawed adipose tissue with the highest MSC concentration and the lowest OR indices were the tissue samples that had been cryopreserved with 0.5 M DMSO + 9% FBS + 0.2 M trehalose. This adipose maintained good morphology, cell proliferation and differentiation potential. Other studies have also confirmed that the combination of non-penetrating CPAs with penetrating CPAs results in better cryopreservation of adipose tissues with more efficient cell survival and greater retention of the volume of adipose tissue (22, 24, 25, 26, 27, 28).

It is well documented that DMSO is toxic to cells (22). The concentrations of DMSO and trehalose for adipose tissue cryopreservation are typically 0.5 M (3.3%) and 0.2 M (7.6%), respectively. Cui et al. demonstrated the best protective effect of 0.5 M DMSO and 0.2 M

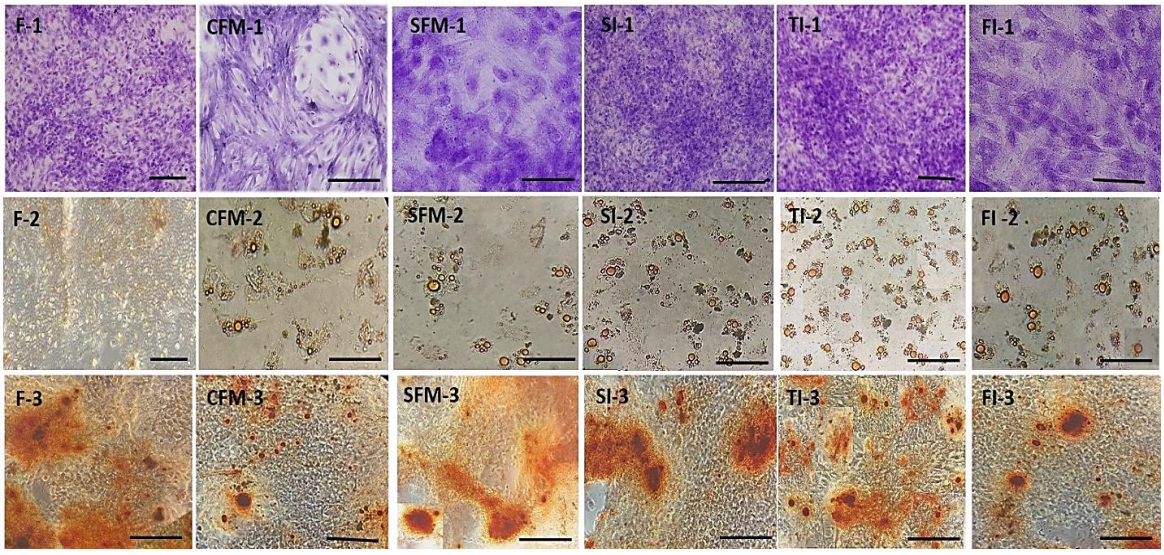

Figure 3. Differentiation potential of mesenchymal stem cells derived from fresh (F) and frozen-thawed (NCG, CFM, FI, TI, SI) adipose tissues into chondrocytes (F-1 to FI-1), adipocytes (F-2 to FI-2), and osteocytes (F-3 to FI-3).

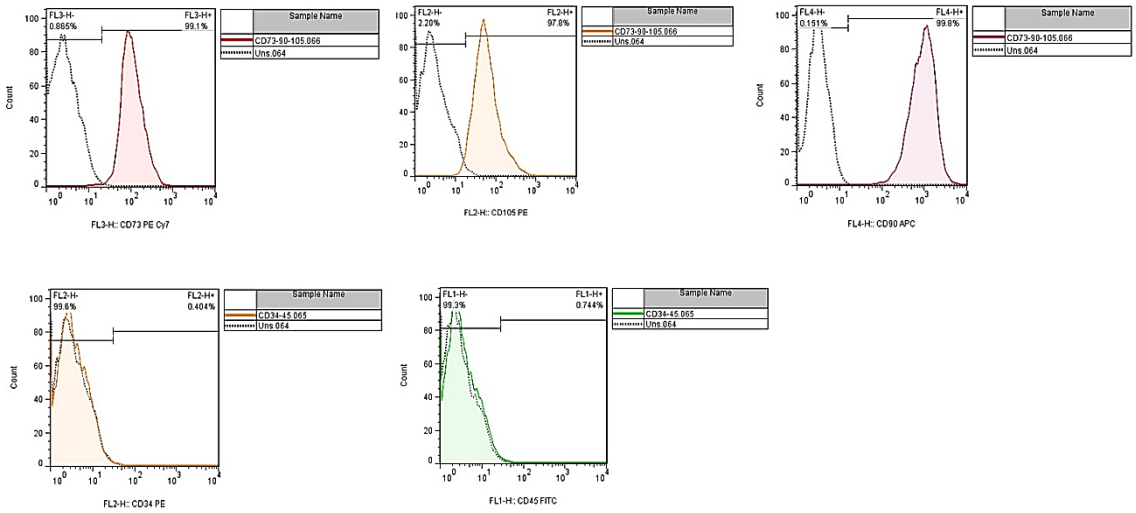

Figure 4. Expression of CD73, CD105, CD90, CD34, and CD45 markers on MSCs derived from adipose tissues cryopreserved with 0.5 M DMSO + 9% FBS + 0.2 M trehalose.

trehalose on the recovery rate of frozen-thawed adipose tissues (29).

In our study, four months after grafting frozen-thawed human adipose tissues into nude mice, frozen adipose tissues cryopreserved with DMSO and trehalose retained significantly more weight and volume of the injected tissue with less tissue fibrosis and shrinkage than the other groups. The combination of DMSO/trehalose formulation, slow freezing and fast melting was used in the present study. Cui et al. evaluated the effect of 0.5 M DMSO + 0.2 M trehalose on the morphological and histological characteristics of adipose tissue transplanted into rats (26). Four months after transplantation, more wrinkles, shrinkage and tissue fibrosis were observed in adipose tissue cryopreserved without CPAs, while the greatest number of viable adipocytes ($2.1 \pm 0.5 \times 10^6$) were detected in the frozen group with DMSO and trehalose. The maximum recovery rate of adipose tissues after freezing was obtained from slow freezing and rapid rewarming (29). Pu et al. confirmed that the DMSO/trehalose combination resulted in more viable adipocytes with better function. After grafting, the retained weight was significantly higher in the DMSO/trehalose group compared to simple freezing (32±8.4% vs. 5±2.7%). It also showed significant tissue fibrosis and fatty degeneration upon simple freezing (30).

Pu et al. showed that lipoaspirates frozen with 0.5 M DMSO + 0.2 M trehalose yielded an average of $3.7\pm1.4\times10^5$ cells/mL, equal to 90% of cells obtained from fresh aspirates ($4.1\pm1.4\times10^5$ cells/mL). However, they reported significantly lower glycerol-3-phosphatase dehydrogenase activity and reduced adipocyte cellular function. Cryopreserved adipose tissue had a suboptimal level of adipocyte-specific enzyme activity compared to fresh grafts and thus may not survive well after transplantation (31). Several studies also showed that the combined use of DMSO and FBS as CPA protects better and obtains greater volume retention after thawing (24, 25, 27, 28, 29). However, Zhang et al. found that the combination of DMSO and FBS increased fibrosis and inflammation in frozen adipose tissues, and recommended 70% glycerol to cryopreserve adipose tissue (12).

In 2009, De Rosa et al. used different CPA compounds and showed that the most suitable combination for fat tissue freezing is 90% FBS, 4% DMSO and 6% trehalose. The thawed cells had high survival and differentiation potential that showed higher expression of surface antigens, close to those observed in freshly isolated cells (32). In another study, adipose tissues were frozen with different concentrations of glycerol (60%, 70%, 80%, 90%, and 100%), trehalose, and combination of FBS and DMSO. The highest number of MSCs derived from frozen-thawed adipose tissue was related to 70% glycerol ($10.3\pm1.3\times10^5$ cells/mL) and FBS + DMSO groups ($11.3\pm3.9\times10^5$ cells/mL). The group without CPAs and those with 60%, 90%, and 100% glycerol showed less viable cells. The histomorphological structure of frozen tissues with trehalose, 60% and 70% glycerol were well preserved. However, the best structural integration of adipose tissues with the most viability and functionality was reported in the group treated with 70% glycerol. They recommended 70% glycerol as an efficient CPA for cryopreservation of adipose tissues (12). Regarding the use of other complex CPAs for preservation of human adipose tissue-derived MSCs, Fujita et al. studied the effects of dextran 40 (4.5%, and 5%), trehalose (3%), DMSO (5%, and 10%) and propylene glycol (2.5%, 4%, and 10%) on survival after freezing. They showed that the combination of 3% trehalose, 5% DMSO and 2.5% propylene glycol was the best cryopreservation solution for adipose tissue and its derived MSCs (33).

However, some studies considered the use of CPAs unnecessary for adipose tissue cryopreservation. Shu et al. did not show a significant difference in the structure of adipose tissue and its cell viability in adipose tissues frozen with and without CPA at -20°C, -80°C or -196°C (5). Roato et al. described that MSCs derived from DMSO-frozen adipose tissue grew faster and reached confluence within days with a better morphology than those treated with trehalose (34). Yong et al. compared the effects of different concentrations of trehalose (0.25 M), DMSO (5%, and 10%), and the combination of FBS and DMSO (20% + 5%, and 90% + 10%) on morphological characteristics, proliferation capacity, differentiation potential, stemness and viability rate of human MSCs-derived adipose tissue (35). Even with a reduction of DMSO to 5% and without FBS, cryopreserved ASCs maintained normal cell phenotype and high proliferation and viability rates, comparable with medium containing 10% DMSO and 90% FBS. Cryopreserved ASCs maintained the capability of differentiation and an enhanced expression level of stemness markers (*e.g.*, NANOG, OCT-

4, SOX-2 and REX-1) (35). The contradictory findings are probably attributed to various reasons such as different adipose tissue harvesting methods, trimming volume, cooling and thawing methods, types and concentrations of CPA, storage temperatures, and pretreatment of adipose tissue before freezing (5, 31, 37, 38, 39).

In conclusion, we recommend the combination of 0.5 M DMSO + 9% FBS + 0.2 M trehalose along with slow freezing and rapid thawing to cryopreserve human adipose tissues.

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