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The Effect of Glycerol as a Cryoprotective Agent in the Cryopreservation of Adipose Tissue

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Abstract

Background: Long-term preservation of adipose tissue is crucial for clinical applications. Researchers should consider both efficiency and biosafety when choosing a cryoprotective agent (CPA) for adipose tissue preservation. Glycerol has been applied as a nontoxic CPA for multiple tissues but not adipose tissue. We aimed to evaluate the efficacy of glycerol as a CPA for adipose tissue cryopreservation.

Methods: Fresh human adipose tissues were obtained from ten patients who underwent liposuction and divided into 1 ml samples. Each sample was randomly mixed with 1 ml of CPA: 60 to 100% glycerol, 0.25 mol/L trehalose or DMSO+FBS and cryopreserved in -196 °C liquid nitrogen for one month. After thawing and elution, the tissues were immediately evaluated for activity and structural integrity in vitro. Then, 0.2 ml of each sample was transplanted subdermally to the nude mouse dorsum and harvested after one month for histological examination to assess the effect of the cryopreserved fat in transplantation.

Results: After cryopreservation, the samples treated with DMSO+FBS, trehalose, and 60% and 70% glycerol had a more integrated structure than the samples in other groups. Tissues preserved with 70% glycerol had the highest tissue activity, close to that of fresh tissues. Adipose-derived stem cells (ADSC) viability, proliferation and differentiation capability were also better in 70% glycerol group. In vivo analysis showed that tissue preserved with 70% glycerol had superior retention rates and structural integrity. Compared to the DMSO+FBS and trehalose groups, the glycerol group showed lower inflammation.

Conclusion: Glycerol (70%) is efficient in adipose tissue cryopreservation. Glycerol-based CPAs, which are nontoxic and show biosafety, are a promising solution for clinical tissue cryopreservation.

1 Background

Autologous fat transplantation is considered an ideal method for soft tissue surgery and repair in plastic and reconstructive surgery due to its bioavailability and biocompatibility[1]. Fat grafting for reconstruction or aesthetic surgery usually requires multiple transplantations to achieve the treatment goals due to its unpredictable absorption rates[2]. Preserving adipose tissue after a single liposuction enables multiple reinjections at the proper time, thus eliminating repeated liposuction, increasing patient acceptance, and reducing costs and suffering. Moreover, adipose tissue contains many adipose-derived stem cells (ADSCs), which have multipotent mesenchymal differentiation potential[3]. Addressing the problem of long-term preservation of adipose tissue can also provide a new solution for stem cell storage.

Cryopreservation has been studied both clinically[4, 5] and experimentally[6, 7] as a solution for tissue and cell preservation. Cryoprotective agents (CPAs) are necessary to reduce the damage caused by ice crystals formed during freezing[8]. Adipose tissue, as a composite tissue, requires CPAs that can effectively preserve multiple cell types, especially adipocytes containing large oil drops. However, in previous studies, researchers often applied traditional cellular CPAs that were either cytotoxic[9] or lacked evidence of efficacy[10].

Glycerol is a molecule that protects cells from freezing injury mainly by reducing intracellular ice crystal formation and osmotic pressure differences[11]. This molecule has been studied as a CPA for composite tissues such as testicular tissue[11], ovarian tissue[12], bones and cartilage[13]. As the backbone of triglycerides contained in adipocytes[14], glycerol has a potential advantage in adipose tissue cryopreservation.

Therefore, in this study, we sought to evaluate the feasibility and efficacy of glycerol-based CPAs for adipose tissue cryopreservation, thus looking for a safe and reliable CPA for future clinical use..

2 Methods

2.1 Harvest and preparation of adipose tissue

Human adipose tissues were obtained from discarded tissue from 5 healthy female patients (aged 21 to 46 years) who underwent abdominal liposuction. Tissues were harvested by low negative pressure liposuction via a 3-mm blunt cannula. Written informed consent was obtained from all patients before surgery. This study was approved by the Ethics Committee of Shanghai Ninth People's Hospital and complied with the principles of the Declaration of Helsinki.

The adipose tissue was washed with phosphate-buffered saline (PBS) to remove free oil and liquid as previously reported[15]. The middle layer of pure adipose tissue was obtained and divided into 1 ml samples (Fig. 1).

2.2 Cryopreservation of adipose tissue

Prepared adipose tissue samples were randomly divided into the following groups: (1)–(5) 60%, 70%, 80%, and 90% glycerol-PBS solutions (volume/volume, V/V) and 100% pure glycerol (Bio-Rad Laboratories, Hercules, USA); (6) 0.25 mol/L trehalose(Solarbio, Beijing, China)-PBS solution as a nontoxic CPA according to a previous study[15]; (7) 90% fetal bovine serum (Gibco Life Technologies, Grand Island, USA) and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA) (FBS + DMSO) (V/V), as a positive control; and (8) no CPA (blank). Each group contained 75 ml of adipose tissue (15 ml from each volunteer, 6 volunteers in all). All the CPAs above were prepared within 30 minutes before use. One milliliter of adipose aspirate was mixed with 1 ml of CPA solution at room temperature in a 5 ml cryogenic vial.

Programmed cryopreservation using a controlled-rate freezing container (Thermo Scientific, Waltham, USA) was applied in this study. Mixed samples were kept in a freezing container at − 80°C for at least 12 hours before transfer to -196°C liquid nitrogen as the standard protocol[7].

2.3 Thawing and elution

After one month of cryopreservation, the samples were removed from liquid nitrogen and immediately placed in a 37°C water bath until they were thoroughly thawed[7]. Five milliliters of PBS was added slowly

to the cryopreserved tissues and mixed for 3 minutes to wash the tissue. The mixed samples were centrifuged at 500 rpm for 3 minutes to separate and remove the liquid in the lower layer. This procedure was repeated twice to thoroughly remove the CPAs.

2.4 G3PDH assay

The G3PDH (glyceraldehyde-3-phosphate dehydrogenase) activity assay kit (MAK208-1KT; Sigma-Aldrich, St. Louis, USA) was applied to assess the cellular activity of adipose tissues[16]. Briefly, tissue samples (10 mg) were homogenized in 200 ml of ice-cold GPDH assay buffer and then centrifuged at 12,000 g for 5 minutes. The supernatant was transferred to a fresh tube and analyzed according to the protocol. Fifty microliters of the appropriate reaction mix were added to each of the wells. The absorbance at 450 nm (A450) per minute was measured in a microplate reader in kinetic mode for 60 minutes at 37°C. The G3PDH activity was calculated according to the reaction curve and equation provided by the manufacturer. The G3PDH activity of fresh tissue was set as a control, and all groups were compared.

2.5 Stromal vascular fraction (SVF) isolation and viability count

The SVF was isolated from 2 ml of cryopreserved adipose tissue as previously described. Thawed and eluted tissue was digested in 0.2% collagenase II (SERVA Electrophoresis GmbH, Heidelberg, Germany) for 30 minutes at 37°C in a shaking air bath and filtered through 40 μm meshes (BD Falcon, Franklin Lakes, United States). Then, the filtered cell suspension was centrifuged (1500 rpm for 3 minutes) and resuspended as an SVF cell suspension. SVF cell numbers and viability were then measured with an automated cell counter (Cellometer Auto 2000; Nexcelom, Lawrence, USA).

2.6 Cell proliferation and differentiation

ADSCs from different groups were subcultured to the 3rd generation and seeded into 6-well plates. The cells were cultured in low-glucose DMEM (Thermo Fisher Scientific, Waltham, USA) containing 10% FBS and 1% penicillin-streptomycin solution (GE Healthcare Life Sciences, Freiburg, Germany) until the cell growth density was 90% of the plate bottom.

A Cell Counting Kit-8 (CCK-8) assay was conducted to examine the proliferative capacity of ADSCs as previously described[17]. Briefly, a total of 10 μl of CCK-8 reagent was added to each well on days 1, 3, 5 and 7. After incubation for 30 minutes at 37°C, absorbance at 450 nm was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, USA). Cell proliferation was further calculated using a standard curve.

Adipogenic differentiation was conducted by changing the culture medium to adipogenic induction medium (Stempro Adipogenic Medium; Cyagen, Santa Clara, USA) followed by culture for 14 days according to the protocol. The results were examined by oil red O staining.

2.7 Fat transplantation model

All animal experiments were performed in accordance with the Animal Use Committee of the Shanghai Jiao Tong University animal guidelines. Glycerol solution (90%) and glycerol (100%) demonstrated poor cryoprotective effects in vitro and were eliminated in the in vivo studies; thus, 6 groups were studied (60% glycerol, 70% glycerol, 80% glycerol, FBS + DMSO, trehalose, and blank). Twenty-four BALB/c male mice (6 weeks old, weighing 25–30 g) (Shanghai Experimental Animal Center, Shanghai, China) were randomly divided into 6 groups (4 mice/8 sides each group).

After anesthesia with 2.5% isoflurane, prepared fat tissue (0.2 mL) was injected into one side of the nude mouse dorsum using a blunt-tip cannula via a 2-mm incision. Each mouse was injected on both sides of the dorsum. After 4 weeks of observation, the animals were sacrificed, and the grafted fat samples were harvested and weighed. Harvested fat samples were cut along the middle point of its long axis, half for histological studies and the other half for RNA extraction.

2.8 HE staining

Cryopreserved samples and fresh tissues were fixed immediately in 4% paraformaldehyde, embedded in paraffin and sectioned at 6 μm for histological examination. Adipocytes, inflammatory cells, tissue fibrosis, necrosis, cysts, and general structure were observed and studied under a microscope (Nikon, Tokyo, Japan). Fibrosis was characterized by the presence of collagen fiber bundles and fibroblasts around adipocyte clusters. The fibrotic area was measured by ImageJ 1.x (National Institutes of Health, USA) [fibrotic area (%) = fibrotic area/total area of the same section*100%]. The vacuole area was characterized by the area of the cysts [vacuole area (%) = vacuole area/total area of the same section*100%].

2.9 Immunofluorescence staining

Adipose tissue sections of all groups were stained with primary antibody against perilipin (1:200, Abcam,Cambridge, United Kingdom). Nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, USA), and sections were mounted on a coverslip. Perilipin is a sensitive and reliable indicator of adipocyte viability because it stains the membrane of living adipocytes. Imaging was evaluated using confocal microscopy (Nikon, Tokyo, Japan).

2.10 RT-qPCR

mRNA extraction and purification were executed by the TRIzol method following a previously reported protocol[18]. RNA integrity and quantity were assessed with a NanoDrop 8000 Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA). For quantitative real-time PCR analysis, 500–2000 ng of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit, SYBR Green PCR master mix and 300 nM primers on an Applied Biosystem StepOne instrument (Thermo Fisher Scientific, Waltham, USA). Relative gene expression was calculated by the ΔΔCT method and normalized to TBP for human analysis. Primers are listed in Table 1. All mRNA expression analyses were performed in a blinded manner.

Table 1
List and sequence of primers

	Forward Primer	Reverse Primer
IL-1	GCCAGTGAAATGATGGCTTATT	AGGAGCACTTCATCTGTTTAGG
IL-6	CACTGGTCTTTTGGAGTTTGAG	GGACTTTTGTACTCATCTGCAC
TNF-α	TGCACTTTGGAGTGATCGGC	GCTACAGGCTTGTCACTCGG
TGF-beta	CTGTACATTGACTTCCGCAAG	TGTCCAGGCTCCAAATGTAG
Caspase-9	CTGCTGCGTGGTGGTCATTCTC	TCGACCGACACAGGGCATCC

2.11 Statistical analysis

All data were collected from at least three independent replications. Experimental data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, USA). One-way analysis of variance (ANOVA) was performed to determine the significant differences among all groups, followed by Tukey's multiple comparison tests as a post hoc analysis. Continuous variables are shown as the mean ± SD. $p < 0.05$ was considered statistically significant.

3 Results

3.1 The 70% glycerol group showed the highest tissue bioactivity in the G3PDH assay

After thawing and elution, the bioactivity of cryopreserved adipose tissue was evaluated by the G3PDH assay and compared with that of freshly harvested adipose tissue. G3PDH activity was 24.76 ± 0.48 in fresh adipose tissue and 24.41 ± 0.70 in the 70% glycerol group. No significant difference was found in the G3PDH activity between these two groups ($p > 0.99$), suggesting well-preserved tissue bioactivity in the 70% glycerol group.

The 60% glycerol (12.23 ± 0.61), 80% glycerol (15.93 ± 0.10), 90% glycerol (18.03 ± 2.47), FBS + DMSO (18.85 ± 1.49) and trehalose (18.38 ± 0.53) groups had moderately preserved G3PDH activity. These groups showed significant differences compared with the fresh group and the 70% glycerol group. The 100% glycerol group (3.49 ± 1.38) and blank group (0.92 ± 0.11) had the lowest G3PDH activity (Fig. 2A).

3.2 The 60% and 70% glycerol, trehalose and F + D groups showed better histological features in vitro than the other

groups

HE staining and histological observation were performed in vitro after thawing and elution (Fig. 2B). After one month of cryopreservation, the preserved adipose tissue showed varying degrees of tissue damage associated with freezing damage. In the CPA groups, the tissues from the DMSO + FBS, trehalose, 60% glycerol, and 70% glycerol groups had a more integrated structure, while those from the 90% glycerol and 100% glycerol groups presented obvious tissue shrinkage and necrosis. Tissues from the blank group showed the most deteriorated structure.

3.3 Stromal cells were better preserved in the 70% glycerol group

SVFs were isolated, and the cell viability results indicated that the 70% and 80% glycerol groups had the highest SVF cell viability (72.67 ± 5.80% and 61.63 ± 3.92%), with no significant difference found ($p = 0.147$). Compared to the 70% group, the FBS + DMSO (48.37 ± 5.53), trehalose (58.83 ± 5.61) and 60% glycerol groups (47.76 ± 4.55) had significantly lower SVF cell viability ($p < 0.001$, FBS + DMSO vs 70% glycerol, 60% glycerol vs 70% glycerol; $p = 0.04$, trehalose vs 70% glycerol). The 90% glycerol (38.6 ± 2.95) and 100% glycerol (33.13 ± 4.96) groups had the worst SVF cell viability (Fig. 3A).

The SVF cell count was also determined (Fig. 3B). The total number of living SVF cells from 2 ml of adipose tissue was the highest in the 70% glycerol group [(10.28 ± 1.13) $x10^5$ cells/ml)] and the FBS + DMSO group [11.3 ± 3.87 ($x10^5$ cells/ml)], with no significant difference. Moreover, the 60%, 90%, and 100% glycerol groups, as well as the blank group, showed significantly lower counts of live SVF cells.

3.4 Fat preserved in 70% glycerol showed better ADSC cell morphology and better-preserved proliferation and adipogenic differentiation than that in other groups

After subcultivation, the adherent ADSCs presented a typical spindle-like shape. The results of the CCK-8 assay indicated that ADSCs from the 70% glycerol group had a higher cell proliferation rate than those from the other cryopreserved groups (Fig. 3C, 3D, S1). After adipogenic differentiation, lipid droplet formation was observed in all 8 groups, suggesting that ADSCs from cryopreserved tissue retained their adipogenic differentiation capacity. There were more lipid droplets in the 70% glycerol group than the other groups, suggesting that the isolated ADSCs from the 70% glycerol cryopreserved tissues had stronger regeneration and differentiation (Fig. 3D, S1).

3.5 Tissues from the glycerol and trehalose groups had higher retention rates after transplantation

Four weeks after fat grafting, the transplanted tissues were harvested. Vessels were observed around the grafted adipose tissue, suggesting successful vascularization (Fig. 4A). The weight of the grafted fat was

measured and calculated as volume retention (Fig. 4B). Tissues from the FBS + DMSO group had a retention rate of 19.52 ± 3.86%, while that in the blank group was 18.74 ± 4.05. These two groups had a similar retention rate ($p > 0.99$), which was significantly lower than that of the other groups. The 60% glycerol (51.87 ± 6.13), 70% glycerol (52.37% ± 7.53), 80% glycerol (47.69% ± 6.89) and trehalose groups (46.11% ± 12.26) all presented higher retention rates, with no significant difference among them (Fig. 4C).

3.6 Adipose tissue preserved in 70% glycerol showed better histological features after transplantation than other tissues

The 70% glycerol group demonstrated a well-preserved tissue structure. The structure of grafted fat in this group was integrated, with few areas of cysts and fibrosis. Adipocytes were plump and well organized. The blank group showed a disorganized tissue structure, in which fibrosis and vacuoles occupied most areas of the grafted tissue instead of normal adipocytes. Other groups generally demonstrated preserved structural integrity. However, among them, the 60% glycerol group showed more cysts; the 80% glycerol group and FBS + DMSO group demonstrated more fibrotic areas. The trehalose groups had a preserved general structure, but interstitial fibrosis was severe (Fig. 5A).

Histological quantification of the grafted cryopreserved tissue was performed. The fibrotic area was 12.70% ± 3.26 in the 70% group, with no significant difference compared with that of the 80% glycerol group (20.02% ± 1.77, $p = 0.958$) and the trehalose group (35.95% ± 4.17, $p = 0.207$). The fibrotic area was larger in the 60% glycerol group (41.70% ± 23.42, $p = 0.048$) and the FBS + DMSO group (47.53% ± 6.88, $p = 0.024$) than in the 70% glycerol group. The blank group had a fibrotic area up to 53.38% ± 16.39 (Fig. 5B).

The vacuole area was also calculated (Fig. 5C). The 70% glycerol group had the lowest vacuole rate (11.35%±3.97). The vacuole rates were moderately higher in the 80% glycerol group (16.49% ± 9.71, $p = 0.920$) and the trehalose group (15.40% ± 7.23, $p = 0.978$), with no significant difference from that in the 70% glycerol group. The other three groups, the 60% glycerol (35.35% ± 10.50), FBS + DMSO (31.49% ± 4.02) and blank groups (30.14% ± 6.41), all had significantly larger vacuole areas.

Anti-perilipin antibody was used to mark mature adipocytes. The results showed that samples from the 70% glycerol group had the most preserved structure of mature adipocytes, which were round in shape and had a continuous membrane. In contrast, perillipin-positive adipocytes were lower in number or lost the oil-containing structures of the other groups (Fig. 6).

3.7 Expression of proinflammatory cytokine genes was lower in the glycerol groups than the other groups

Quantitative PCR analyses were performed to further evaluate the inflammatory and apoptotic activities of grafted adipose tissues. Overall, the expression of the proinflammatory cytokines IL-1, IL-6 and TNF-α was higher in the FBS + DMSO group than in the glycerol groups. Among all glycerol groups, the 70% glycerol group showed the lowest level of proinflammatory gene expression. The relative expression of IL-

1 and TNF-α in the fat grafts from the 70% glycerol group was significantly lower than that in the fat grafts from the 60% and 80% glycerol groups. The expression of IL-1, IL-6 and TNF-α in the trehalose group was similar to that in the 70% glycerol group (Fig. 7A-C).

The expression of Caspase-9 was significantly higher in the blank group than in the other groups. No significant difference was found in Caspase-9 expression among the glycerol groups, trehalose group and FBS + DMSO group (Fig. 7D).

4 Discussion

Proper storage of harvested adipose tissue after thawing and grafting avoids repeated liposuction procedures, thus increasing patient acceptance, reducing patient suffering, and saving time and costs[19]. Current studies regarding the efficacy and reliability of adipose tissue cryopreservation are controversial[4, 5, 20]. Lack of proper CPA is the main obstacle.

Cryopreservation of isolated cells or uniform tissues has been widely studied and applied. However, cryopreservation of composite tissues is a challenge. Different cells in the tissue may have various cryopreservation requirements[13]. Because the structure of the tissue is integrated, it is more difficult for CPAs to infiltrate the tissue thoroughly, especially for adipose tissue.

Several studies have reported that using DMSO or DMSO + FBS as CPAs was effective in protecting adipose tissue cell survival and retention of the adipose tissue volume compared with the absence of CPA[9, 21–24]. However, the use of DMSO and FBS has the risk of toxicity and zoonotic infection[25]. When these agents are applied in tissue cryopreservation, especially in adipose tissue that is grafted into the human body in a large volume, toxicity may become a major concern[26–28]. Furthermore, due to the integrity and complexity of tissue compared with isolated cells, thorough elution of DMSO may be difficult[28, 29]. FBS, as a xenogeneic antigen, may cause severe inflammation of grafted tissue or even allergic reactions[30]. These defects were also proven in our study by histological examination, where the DMSO + FBS group had increased fibrosis and inflammation after grafting in animal models was performed. To address these problems, researchers need a nontoxic, nonimmunogenic and easily available CPA for adipose tissue cryopreservation.

Our study revealed that glycerol at an optimal concentration is an effective agent for adipose tissue cryopreservation. Among all concentrations tested, 70% glycerol solution appeared to be most potent in preserving tissue bioactivity. The tissue from the 70% glycerol group showed comparable activity with fresh tissue when assessed by the G3PDH assay. In vivo studies also demonstrated that the cryopreserved adipose tissue from the 70% and 80% glycerol groups had a higher retention rate and less inflammation and fibrosis after transplantation than the adipose tissue from the DMSO + FBS group. This study also showed that groups with decreased tissue activity had more in vivo fibrosis and inflammation, which is consistent with previous studies showing that oil cyst formation, chronic inflammation, and progressive calcification are the results of fat necrosis[31]. Moreover, the SVF isolated from adipose tissue

contains a large number of ADSCs[24]. The quantity and activity of SVF cells may explain the preserved volume and structure after grafting.

The mechanism underlying the advantages of glycerol in adipose tissue preservation may be as follows: compared with other tissues, adipose tissue has unique characteristics because it is mainly comprised of adipocytes, which contain large amounts of triglycerides. The chemical similarity of glycerol and triglyceride may help cryopreservation. Studies have shown that glycerol, as the backbone of triglycerides stored in adipocytes, is transported outward through glycerol transporters[14]. With an increased concentration of glycerol in the extracellular matrix, glycerol efflux might be inhibited, thus better preserving the structure and bioactivity of adipocytes[32]. Moreover, glycerol is nontoxic and generally nonallergenic to cells and tissues, characteristics that make it suitable for clinical use[13].

We evaluated the cryopreserved tissue morphology, bioactivity and immunohistochemical staining. Histological observation was critical to determine the structural features of the tissue. Immunofluorescence provided further information on cell viability and plasma membrane continuity. The G3PDH assay was conducted on the entire tissue without digestion to examine the bioactivity of the tissue instead of isolated cells based on previous studies[19, 33, 34]. Our results showed that when tissue bioactivity deteriorated, increased levels of fibrosis and inflammation were observed after transplantation. Although the volume retention rate did not decrease sharply, the integrity of the adipose tissue structure was destroyed, with a large area of oil cysts and fibrosis[19].

As previously reported, cryo-damage to cells and tissue occurs mainly during freezing. The most influential factor for tissue activity was the process of freezing and not the duration of storage[35]. Therefore, in this research, we evaluated adipose tissues cryopreserved for 1 month. A longer cryopreservation duration may be required to confirm the long-term preservation effect of glycerol. Although glycerol is nontoxic and preclinical research has shown good efficacy, further clinical trials are required to evaluate its clinical efficiency.

5 Conclusions

Our study demonstrated that glycerol solutions can serve as CPAs for long-term cryopreservation of adipose tissue. Among all concentrations studied, 70% glycerol solution was superior at preserving adipose tissue bioactivity, reducing the retention rate, and preventing tissue fibrosis and inflammation in cryopreserved adipose tissue. Furthermore, glycerol is a nontoxic and nonimmunogenic agent and thus is safer and more compatible with clinical use than other CPAs. Future studies are required to further prove the safety and optimize the reliability of adipose tissue cryopreservation using this method.

List Of Abbreviations

ADSC	Adipose-derived stem cell
CCK8	Cell Counting Kit-8
CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai Ninth People's Hospital and complied with the principles of the Declaration of Helsinki. All participants provided written informed consent for the scientific use of aspirated adipose tissue. All animal experiments were performed in accordance with the Animal Use Committee of the Shanghai Jiao Tong University animal guidelines.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Authors' contributions

PQZ, SBZ and QFL initiated and designed the study and protocol. PQZ and PCT conducted experiments. PQZ and PCT contributed to data collection, data analysis and data interpretation. YMG, XJZ, and DNZ. YX and SBZ performed liposuction surgeries. PQZ wrote the first draft of the manuscript. SBZ and QFL critiqued and modified the manuscript. All authors reviewed and approved the work.

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Figures

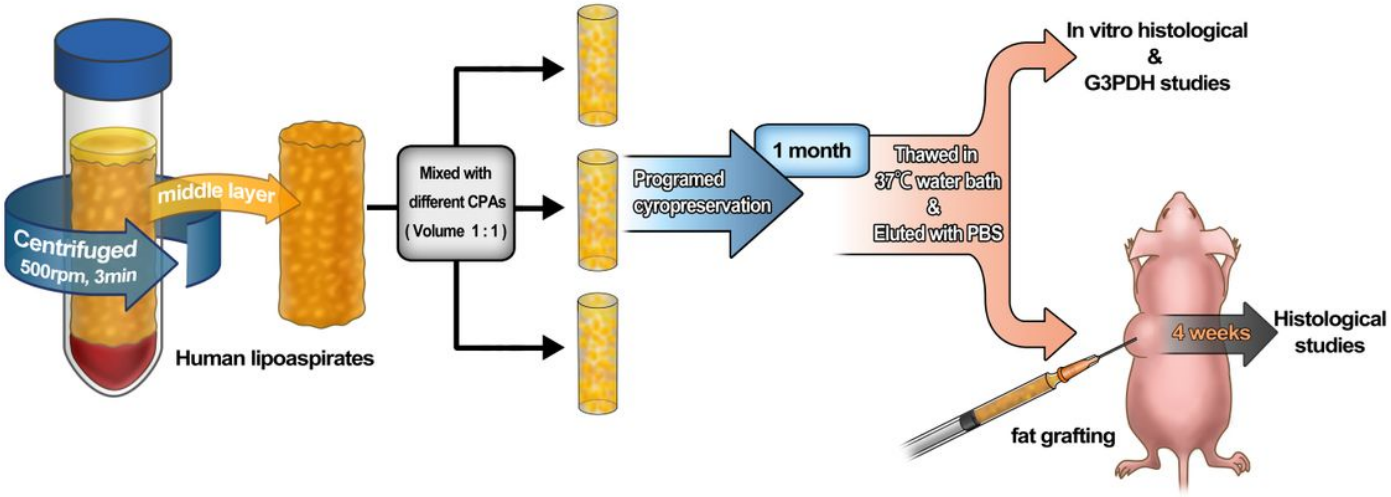

Figure 1

Schematic representation of the cryopreservation and experimental methods. Aspirated fat was centrifuged at 500 rpm for 3 minutes. The middle layer of adipose tissue was mixed with different

cryoprotective agents (CPAs) at a 1:1 volume ratio. The mixtures were then cryopreserved with a programmed protocol and stored in liquid nitrogen at −196 °C. After 1 month of cryopreservation, the tissues were thawed and eluted. In vitro and in vivo studies were then conducted.

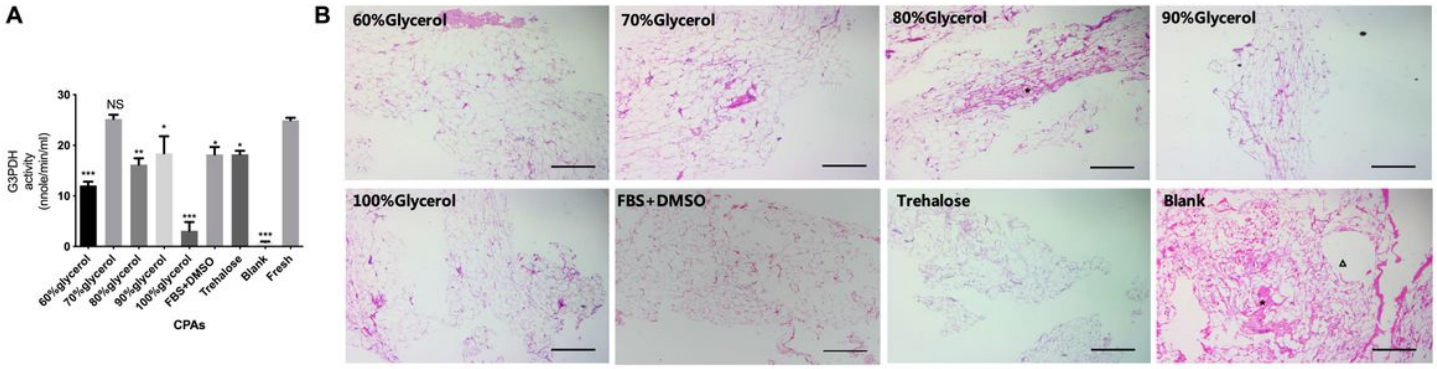

Figure 2

Bioactivity and morphological assay of the cryopreserved adipose tissue. (A) G3PDH assays in the CPA groups. All CPA groups were compared with fresh tissue. No significant difference was found between the 70% glycerol and fresh groups. (B) Images show HE-stained fat graft sections of 8 groups one month after fat grafting. All samples were cryopreserved at -196 °C for 1 month. Necrosis (*) was obvious in the blank group and the 80% glycerol group. A cyst (△) is shown in the blank group. Scale bar: 250 μm. *p<0.05, **p<0.01, ***p<0.001.

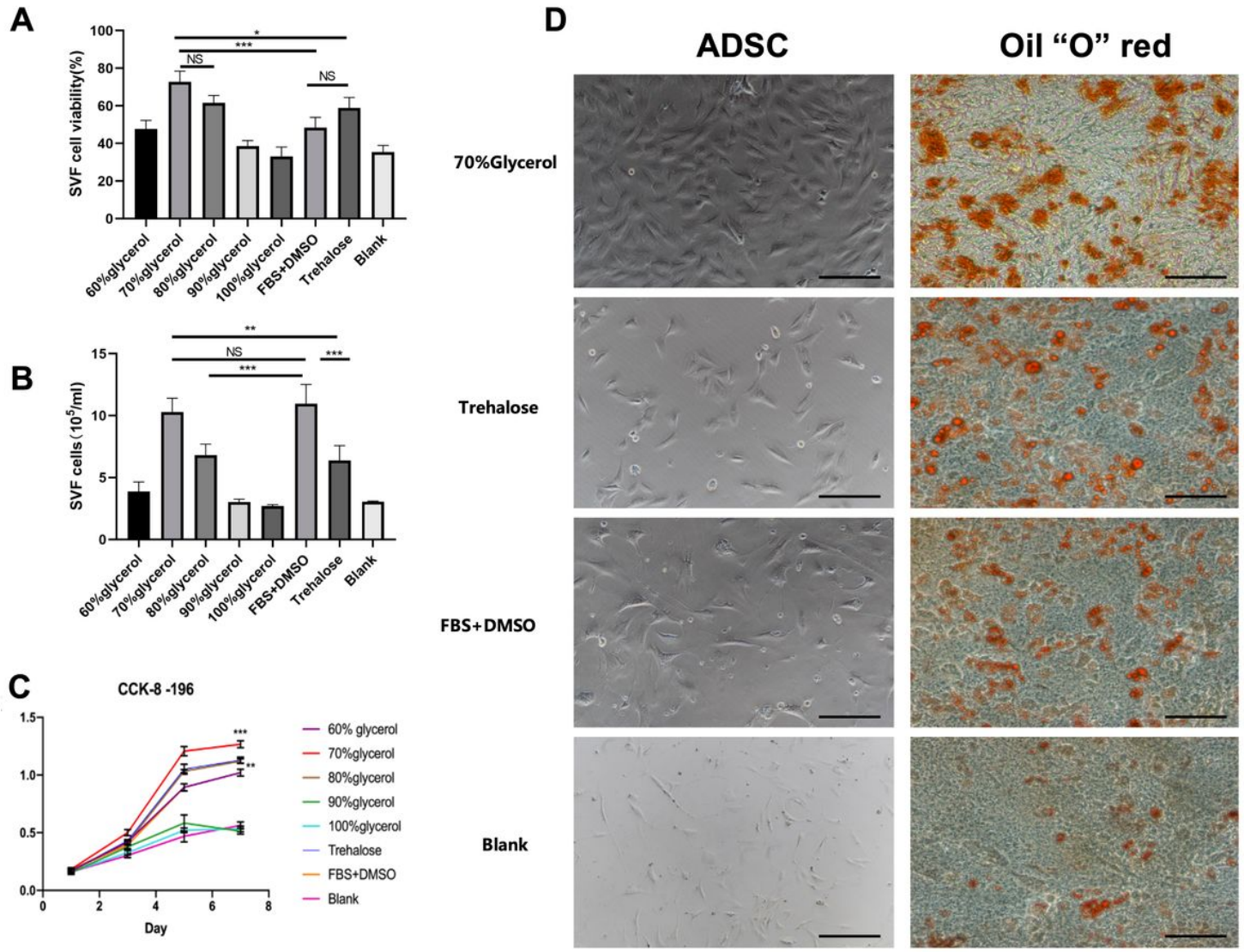

Figure 3

Stromal vascular fraction (SVF) cell viability and function. (A) Viability of the SVF cells. ***p<0.001 (70% glycerol vs FBS+DMSO), *p=0.040 (70% glycerol vs trehalose), NS (70% vs 80% glycerol, FBS+DMSO vs trehalose). (B) The total number of SVF cells obtained from 2 ml of adipose tissue from different CPA groups. **p=0.0015 (70% glycerol vs trehalose), ***p<0.001 (80% glycerol vs FBS+DMSO, FBS+DMSO vs trehalose), NS (70% glycerol vs FBS+DMSO). (C) CCK-8 assay. ***p<0.001 (70% glycerol vs blank), **p<0.01 (trehalose vs blank). (D) Under the same culture conditions, the growth density of the cells in the 70% glycerol group was higher than that in the other groups, and the cell morphology was significantly better, indicating that the cell proliferation was maintained well after cryopreservation. Oil red O staining showed that the 70% glycerol group had more red lipid droplets, while the other groups had fewer red lipid droplets, suggesting that the adipogenic differentiation ability of adipose-derived stem cells in the 70% glycerol group was stronger. Scale bar: 250 μm. *p<0.05, **p<0.01, ***p<0.001.

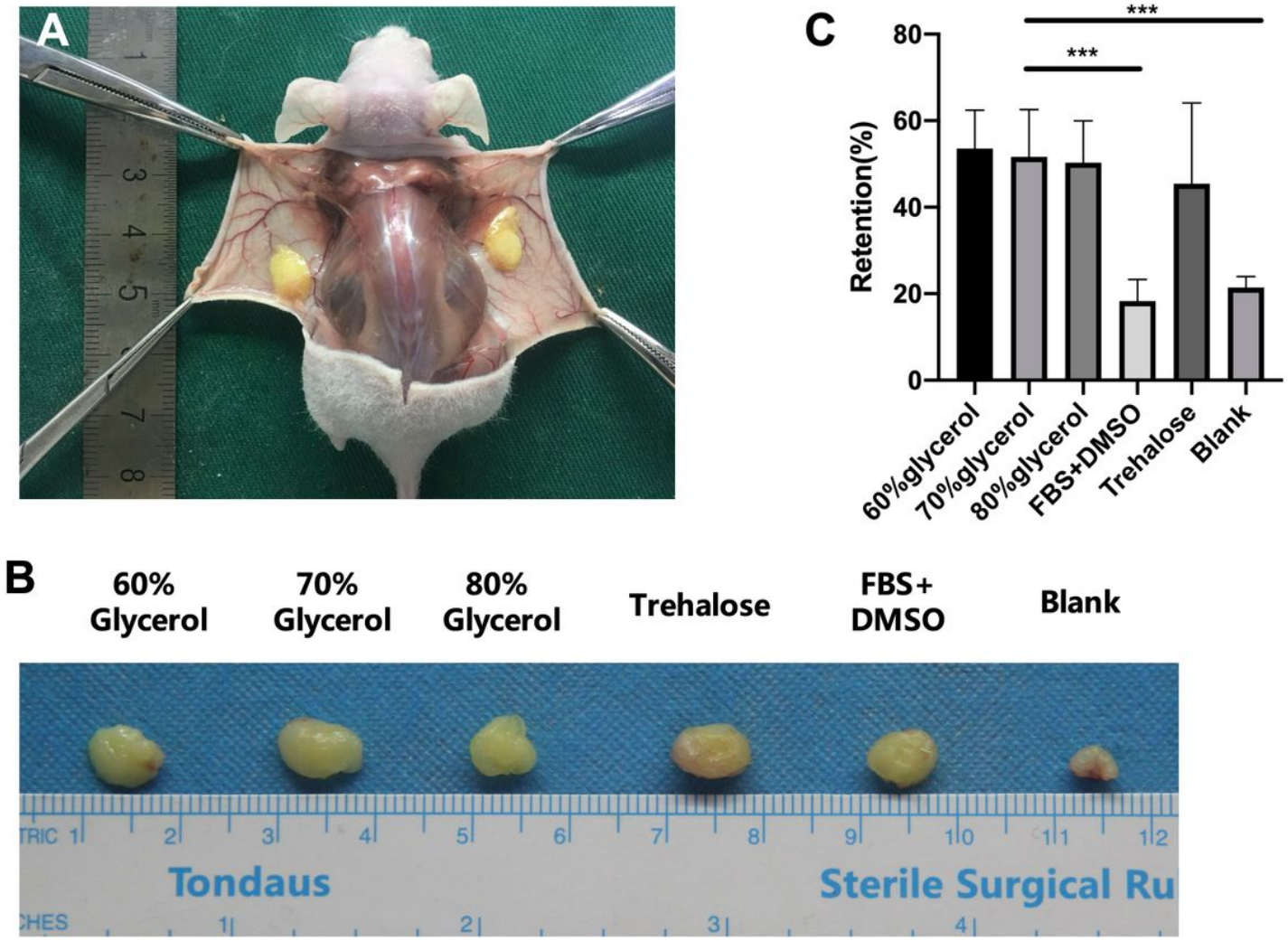

Figure 4

Grafted fat harvested four weeks after transplantation. (A) Fat graft analysis using nude mice. Vascularization was found to be associated with grafted fat. (B) Volume comparison of the grafted fat samples. (C) Retention rate of the CPA groups. *p<0.05, **p<0.01, ***p<0.001.

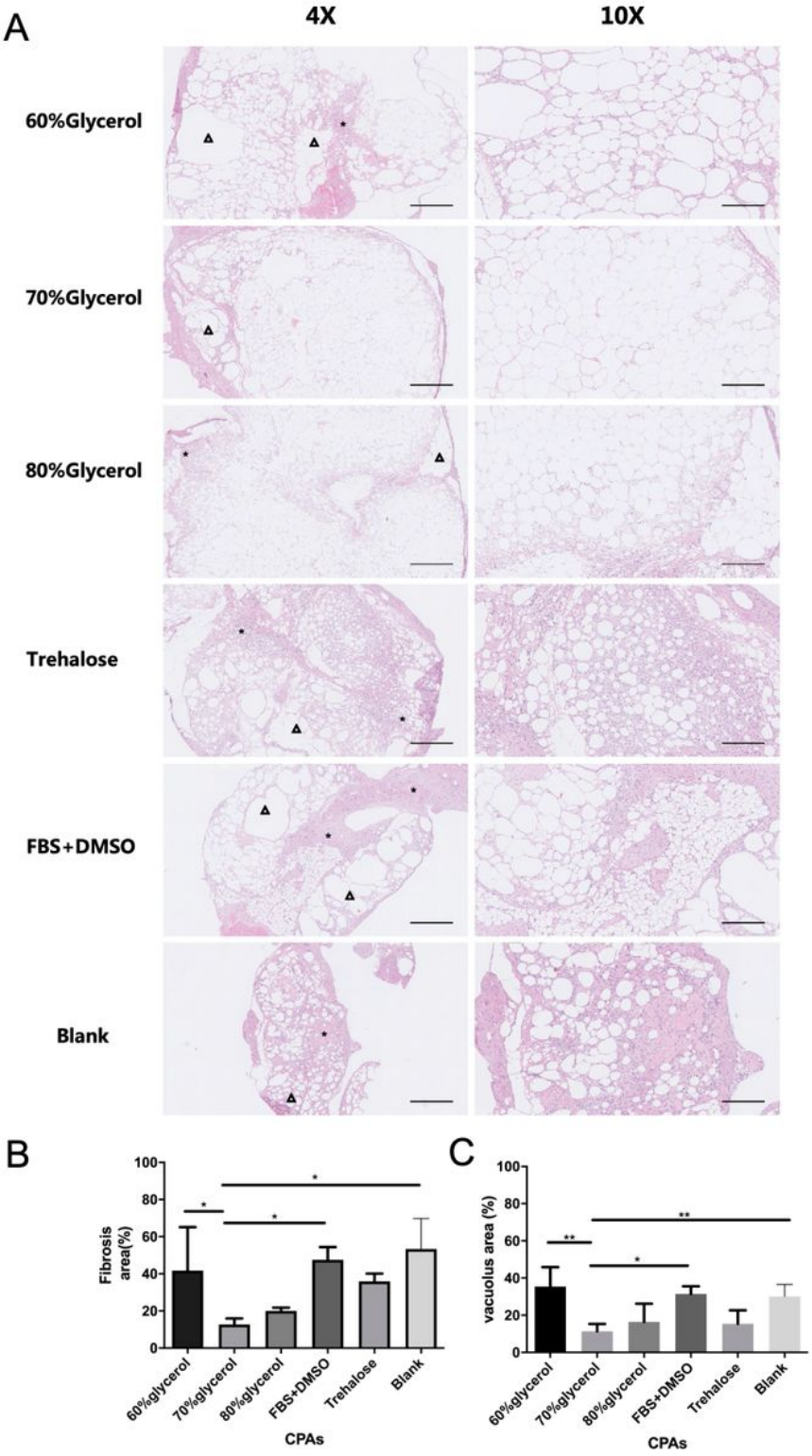

Figure 5

Adipose tissue histology after fat graft. (A) Representative images of HE-stained fat grafts four weeks after grafting. Preserved tissue construction and integrity were observed in the 60%, 70% and 80% glycerol groups, while the 60% glycerol group had more obvious vacuole cyst (△) formation. The trehalose group demonstrated apparent fibrosis and inflammatory infiltration (*). Fibrosis, inflammation (*) and vacuole formation (△) were detected in the FBS+DMSO group. The blank group demonstrated a

disorganized structure, fibrosis and inflammatory (*) infiltration. (Scale bar: 250 μm.) (B) The fibrotic areas of grafted fat samples . (C) The vacuole areas of grafted fat samples . *p<0.05, **p<0.01, ***p<0.001

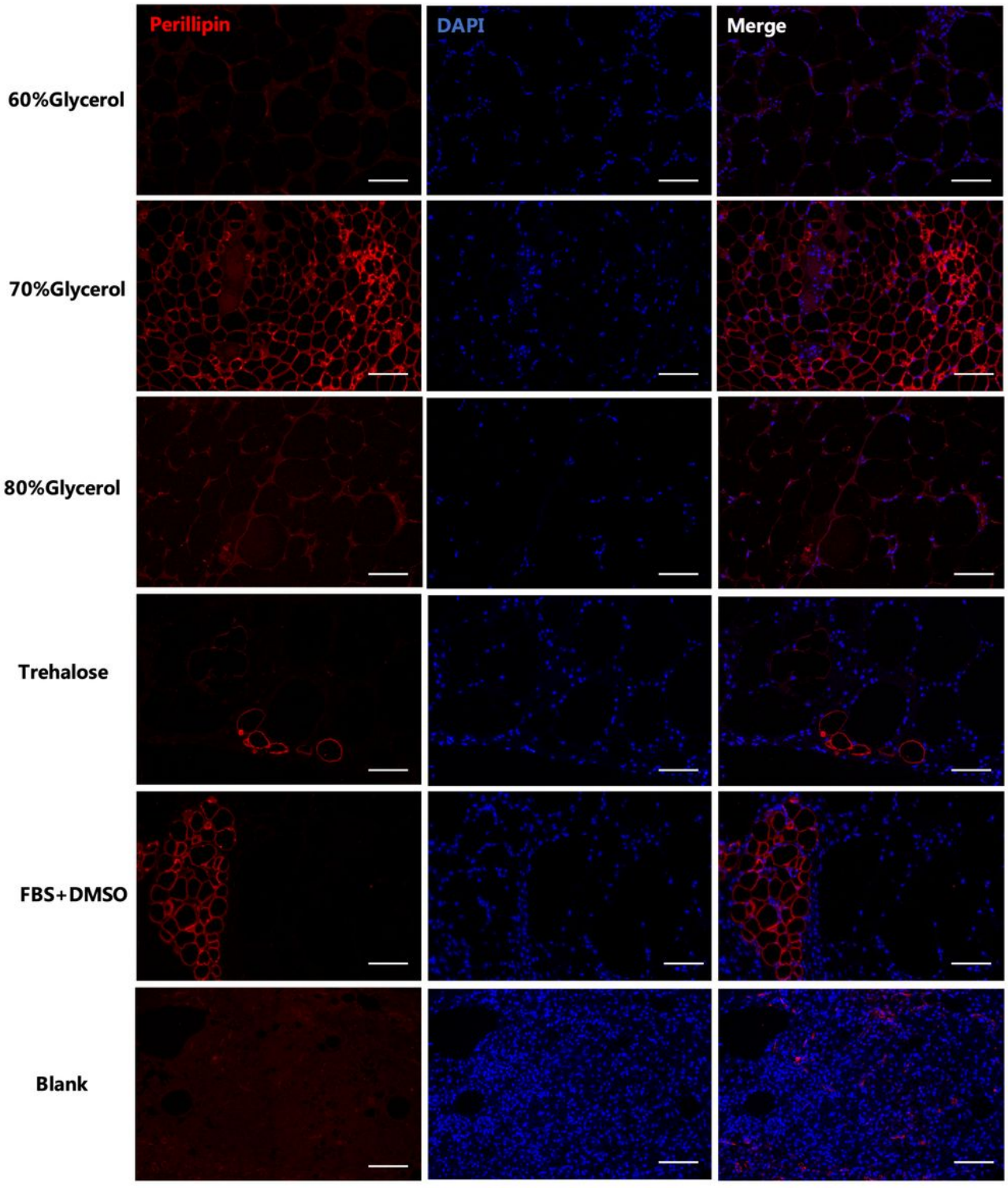

Figure 6

Immunofluorescence staining of grafted adipose tissue. Anti-perilipin antibody was used to mark mature adipocytes in grafted fat 4 weeks after transplantation. Red, perilipin for viable adipocytes; blue, for

nuclei. Scale bars = 125 μm.

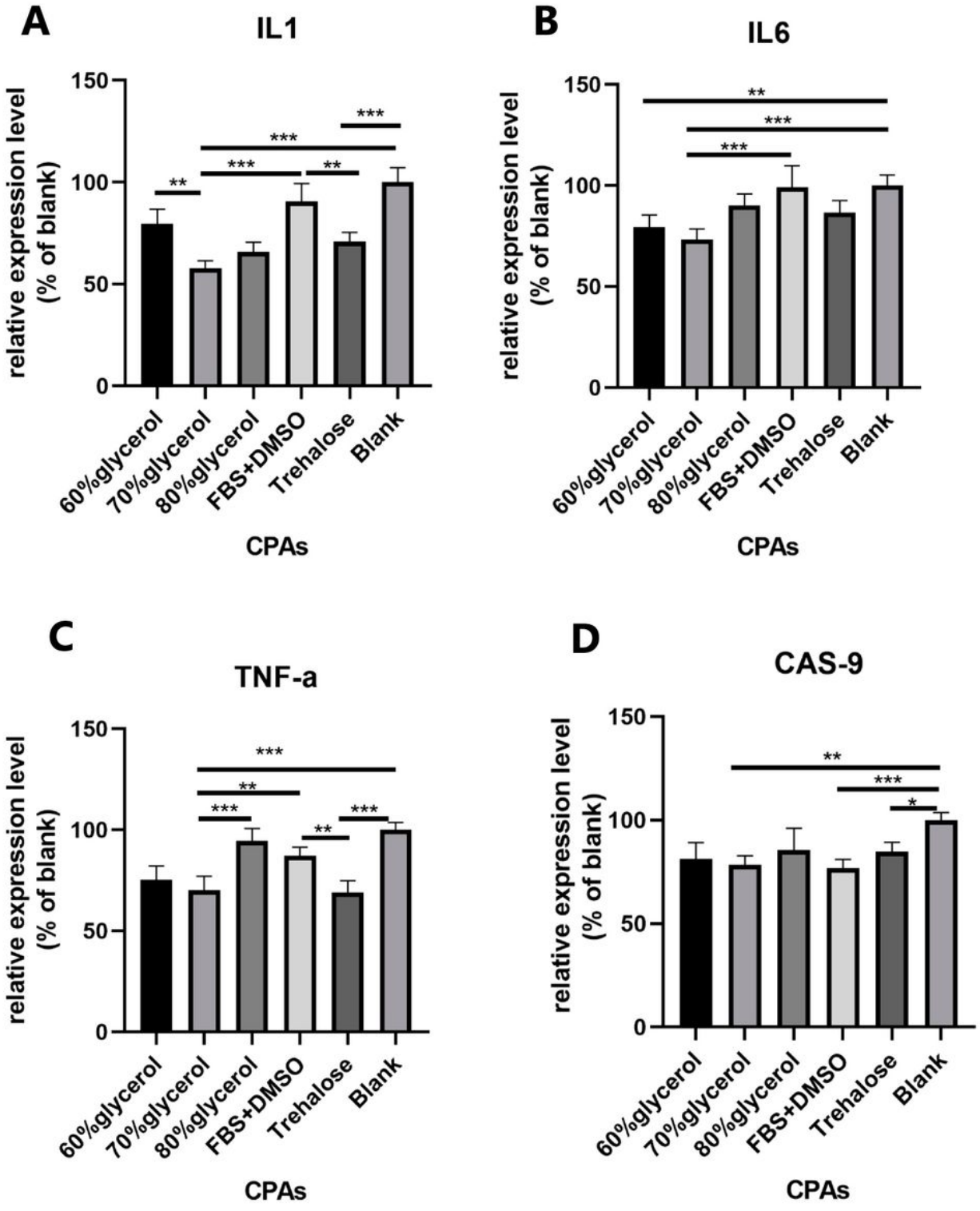

Figure 7

Proinflammatory and apoptotic markers in harvested adipose tissues 1 month after fat graft. The plot shows the relative expression levels of (A) IL-1, (B) IL-6, (C) TNF-α and (D) Caspase-9. *p<0.05; **p<0.01; ***p<0.001.

Supplementary Files

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