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Original Article

Investigation of the stemness and wound-healing potential of long-term cryopreserved stromal vascular fraction cells

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ABSTRACT

Introduction: Stromal vascular fraction (SVF), a heterogeneous cell population primarily derived from adipose tissue, is widely utilized in regenerative therapies for its wound-healing properties and accessibility. While its immediate availability is advantageous, repeated harvesting can be burdensome, especially for elderly patients, and the regenerative capacity of SVF declines with donor age. Long-term cryopreservation offers a potential solution by allowing the banking of SVF from younger donors for future use; however, the impact of this process on SVF functionality remains elusive. This study investigates the stemness and wound-healing potential of SVF following prolonged cryopreservation.

Methods: SVF cells were isolated from adipose tissue harvested from twelve patients and cryopreserved for either two months (short-term cryopreserved SVF, S-SVF) or 12–13 years (long-term cryopreserved SVF, L-SVF), with six patients in each group. In vitro assays assessed cell viability and stemness, while in vivo assays evaluated wound-healing ability by administering thawed SVF cells from each group to dorsal wounds in immunodeficient mice, compared with a control group. Non-parametric statistical tests analyzed the differences between groups.

Results: L-SVF exhibited significantly lower stemness compared to S-SVF. Importantly, the L-SVF group showed significantly improved wound healing compared with the control group, although the wound-healing effect of L-SVF was inferior to that of the S-SVF.

Conclusion: This study demonstrated that, despite reduced stemness, L-SVF retains partial wound-healing potential after 12–13 years of cryopreservation. These findings highlight the need for optimized cryopreservation protocols to enhance SVF viability and regenerative capacity for clinical applications.

1. Introduction

Adipose-derived stem cells (ADSCs) are multipotent cells isolated from adipose tissue that can differentiate into various cell types, such as osteocytes, chondrocytes, and adipocytes. These cells have gained attention as a promising material for regenerative therapy due to their abundance and accessibility [1–3]. Moreover, ADSCs play crucial roles in immunomodulation and angiogenesis, thereby enhancing their therapeutic potential in tissue repair [4].

The stromal vascular fraction (SVF) is a heterogeneous cell population primarily derived from adipose tissue, comprising not only ADSCs but also endothelial cells, pericytes, immune cells, hematopoietic cells, fibroblasts, and other cell types [5–7]. In clinical settings, SVF provides a key advantage over ADSCs due to its immediate availability, as it does not require the isolation, expansion,

Abbreviations: SVF, stromal vascular fraction; ADSCs, adipose-derived stem cells; L-SVF, long-term cryopreserved stromal vascular fraction; S-SVF, short-term cryopreserved stromal vascular fraction.

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and differentiation stages necessary for ADSCs [8]. Studies suggest that this diverse cell population offers superior tissue regeneration capabilities compared to ADSCs alone, attributed to the synergistic effects of its multiple cell types [9]. Consequently, SVF is frequently used in clinical applications related to wound healing and graft survival, leveraging its cost-effectiveness, favorable regulatory profile, and enhanced tissue regeneration potential [5–7,10–13].

Despite these advantages, several challenges arise with SVF use. A single harvest typically yields limited amounts of SVF, often necessitating repeated invasive procedures to obtain adequate cell numbers for therapeutic applications [14–19]. Another concern is that the regenerative capacity of SVF declines with donor age, limiting its effectiveness for elderly patients [20,21]. Long-term cryopreservation offers a potential solution by enabling patients to bank SVF obtained at a younger age for future use, thus circumventing the need for repeated harvesting and potentially enhancing regenerative outcomes. Furthermore, establishing cell banks of allogeneic SVF would support broader therapeutic applications; however, both approaches require effective long-term cryopreservation techniques.

While studies have shown that short-term cryopreserved SVF retains its wound-healing potential, there is limited research on the effects of long-term cryopreservation on SVF's functional characteristics and in vivo efficacy [22]. Most existing studies focus on short-term storage durations, generally from a few weeks to several months, leaving a substantial gap in understanding how prolonged cryopreservation impacts SVF viability, stem cell properties, and therapeutic effectiveness in clinical applications. The mechanisms by which extended cryopreservation might affect SVF properties, such as ice crystal formation, osmotic stress, and oxidative damage, remain largely unexplored.

Addressing these gaps is essential to verify the safety and efficacy of using long-term cryopreserved SVF in regenerative medicine. Successfully preserving the regenerative potential of SVF over extended periods could transform personalized medicine approaches, enabling individuals to bank their own cells for future use and potentially expanding the availability of allogeneic treatments.

In this study, we hypothesized that long-term cryopreservation (12–13 years) significantly reduces the stemness and regenerative potential of stromal vascular fraction (SVF) compared to short-term storage (2 months). Our research uniquely examines an unprecedented cryopreservation duration for SVF, extending well beyond the timeframes explored in previous studies. We aimed to assess specific stem cell characteristics, including viability, proliferation capacity, surface marker expression, and multi-lineage differentiation potential, alongside the wound-healing capabilities of SVF after both long-term and short-term cryopreservation. This comprehensive assessment will provide crucial insights into the feasibility of long-term SVF banking and its potential clinical applications, particularly for regenerative therapies in aging populations.

Table 1
Patient summary.

Group	S-SVF (n = 6)	L-SVF (n = 6)	
Age (years)	50.5 (47.5–57.25)	53.5 (48.75–63.25)	P = 0.6291
BMI (kg/m^2)	21.85 (20.625–22.875)	23.05 (21.7–26.625)	P = 0.146

Values are medians with IQRs.
BMI; body mass index.

2. Materials and methods

2.1. Isolation of SVF cells

Residual aspirated adipose tissue of the abdomen, which would have otherwise been discarded during breast reconstruction, was donated by twelve patients (Table 1). Each donor granted prior informed consent, conforming to the protocol approved by the institutional ethical committee (RBMR-C-631 and ERB-C-2193-1). To isolate SVF cells, the lipoaspirates were digested using Celase® at a concentration of 18 μl per 1 ml of adipose tissue in phosphate-buffered saline at 37 °C for 30 min. The mature adipocytes and connective tissue were separated from the SVF pellets by centrifugation at 200*g* for 4 min (Fig. 1).

2.2. Cryopreservation, preservation and thawing of cells

Approximately 1 × 10^6 cells of the SVF pellets were cryopreserved in one cryovial with 10 % Dimethyl Sulfoxide (DMSO). The cells were initially frozen at −80 °C reducing the temperature gradually, then transferred to a liquid nitrogen tank 24 h later. Cells were divided into two groups for preservation: one for two months (short-term cryopreservation group: S-SVF group) and another for

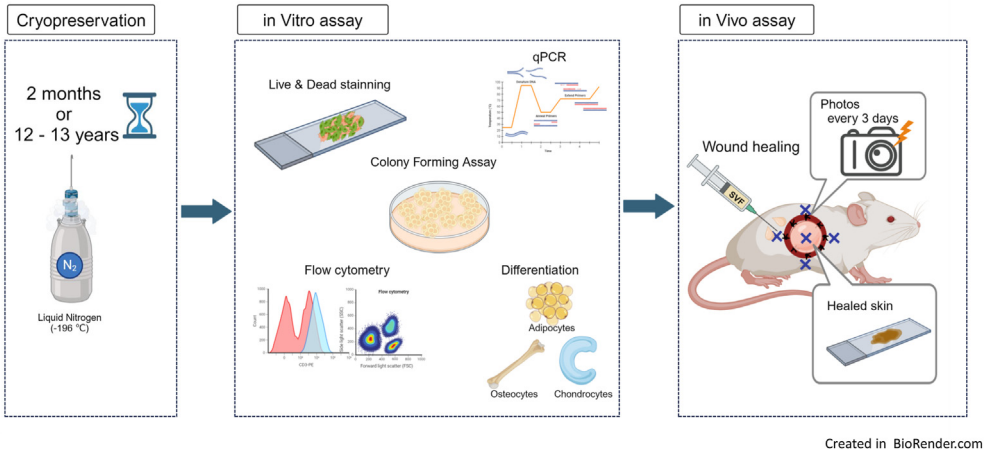

Fig. 1. Experimental design and process. Timeline illustrating the experimental setup. The isolated SVF cells from patients were divided into two groups: one underwent short-term cryopreservation (2 months) and the other long-term cryopreservation (12–13 years) (n = 6 each). Both groups were subjected to in vitro and in vivo assays. In Vitro Assay, assessments were performed for viability and stemness including proliferation, cell surface markers, gene expression, and differentiation of each SVF group. In Vivo Assay for wound healing, SVF cells from each group were administered to mice in wound models and compared to a control group that received no injections. Photographs of the healing areas were taken every three days. Histologic assessments of the healed skin were performed on the 15th day.

12–13 years (long-term cryopreservation group: L-SVF group). After storage, cells were thawed in a 37°C-water bath for 2 min. Following thawing, cells were centrifuged for 2 min after dilution in culture medium, and then seeded in culture dishes with medium containing high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, and 100 mM nonessential amino acids. The cells were maintained at 37 °C in a 5 % CO2 atmosphere in a cell culture incubator. The medium was changed every third day, and cells were passaged once at subconfluence (Fig. 1). The following Live and Dead staining and wound-healing assessment experiments were performed immediately after thawing and washing SVF cells, without cell expansion.

2.3. Live and dead staining (Cell viability assay)

Immediately after thawing, SVF cells were stained for live and dead cells by Live-Dead Cell Staining Kit (BioVision Inc., Milpitas, CA, USA). The thawed cells were centrifuged at 1200 rpm for 1 min and suspended in the culture medium. Subsequently, the cells were resuspended with Staining Solution and incubated for 15 min at 37 °C. The stained cells were observed using a microscope (BZ-X710; Keyence, Osaka, Japan), where live cells appeared green fluorescent and dead cells appeared reddish-orange fluorescent. The rate of viable cells was counted in five randomly selected fields of view at 400× magnification.

2.4. Colony forming assay

The cultured cells were detached by trypsin/EDTA (Nacalai Tesque, Kyoto, Japan) from dishes, and cells were seeded into 24-well culture plates at a density of 5000 cells per well. Colonies were formed after being cultured for 21 days with the 10 % FBS, DMEM and the same supplements as above, and the number of colonies was counted. The colonies were fixed with 4 % paraformaldehyde, stained with Crystal violet solution (Sigma-Aldrich, St. Louis, USA), and counted with a microscope (BZ-X710; Keyence, Osaka, Japan).

2.5. Flow cytometry

The following antibodies were used for flow cytometry staining: phycoerythrin- (PE-) conjugated mouse anti-human CD44 (dilution = 1: 25) (BioLegend, San Diego, CA, USA), PE-conjugated mouse anti-human CD90 (1:25) (BioLegend). The cultured cells for seven days were dissociated as described above, and resuspended in phosphate-buffered saline (PBS) containing 0.5 % bovine serum albumin (BSA), 0.01 % NaN3, and 1 mM EDTA (FACS Buffer), and incubated with the antibodies for 20 min on ice. Cells were washed with FACS Buffer, and flow cytometric analysis was performed using FACSCalibur (BD Biosciences) and CellQuest software.

2.6. Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR analysis was conducted following a standard procedure previously described in the literature [23]. Initially, cultured cells were lysed using Buffer RLT (Qiagen, Hilden, Germany), and total RNA was extracted via the phenol-guanidinium acid method using the QIAcube system (Qiagen). The RNA was then converted to cDNA with the ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantification of mRNA levels was performed with the StepOnePlus Real-Time PCR System (Applied Biosystems, Bedford, MA, USA), utilizing TaqMan® Fast Advanced Master Mix (Applied Biosystems), and specific primers and dye probes listed in Supplementary Table S1. Reaction volumes were set to 20 μl, comprising 2 μl of cDNA (100 ng), 1 μl of each primer and probe, and 10 μl of Master Mix. The thermal cycling conditions included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The comparative threshold cycle (CT) method was used to calculate relative mRNA levels, with GAPDH serving as the normalization reference.

2.7. Differentiation

2.7.1. Adipogenic differentiation

Cells were cultured in adipogenic medium containing α-MEM with 10 % FBS and 1 % penicillin-streptomycin, supplemented with hydrocortisone, isobutyl methylxanthine, and indomethacin for 14 days. After induction of differentiation, qualitative evaluation of cells was conducted with Oil red O staining, and quantitative evaluation was conducted by measuring FABP gene expression with qRT-PCR.

2.7.2. Osteogenic differentiation

Cells were cultured in osteogenic medium containing D-MEM/F-12 with 1 % penicillin-streptomycin, supplemented with dexamethasone, ascorbate-phosphate, and β-glycerol phosphate for 21 days. After induction of differentiation, qualitative evaluation of cells was conducted with Alizarin Red S staining, and quantitative evaluation was conducted by measuring Osteopontin gene expression with qRT-PCR.

2.7.3. Chondrogenic differentiation

Cells were cultured in chondrogenic medium containing D-MEM/F-12 with 1 % penicillin-streptomycin, supplemented with dexamethasone, ascorbate-phosphate, proline, pyruvate, and TGF-β3 for 21 days. After induction of differentiation, qualitative evaluation of cells was conducted with Safranin O staining, and quantitative evaluation was conducted by measuring Aggrecan gene expression with qRT-PCR.

2.8. Wound healing assessment

2.8.1. Wound closure rate

All experimental procedures using animals were performed in accordance with relevant guidelines and approved by the Animal Experimental Committee of the Kyoto Prefectural University of Medicine, Kyoto, Japan. Eight-week-old female immunodeficient mice were selected for the study. A circular full-thickness skin defect, 9 mm in diameter, was created in the dorsal region of each mouse under general anesthesia (sevoflurane inhalation). To prevent wound contracture, donut-shaped silicone splints were set and sutured with 6–0 nylon around the skin defects. Immediately after thawing the cryopreserved SVF cells, six mice in each of the three test groups (18 mice total) were administered each product subcutaneously on all four sides and the central of the circular wound as follows: control group, no administration; S-SVF group, 1×10^5 cells total of 2 month-preserved SVF cells suspended with 1 ml of the same culture medium as above; and L-SVF group, 1×10^5 cells total of 12–13 year-preserved SVF cells suspended with 1 ml of the same culture medium as above. The wounds were covered with nonadhesive dressings, wrapped in transparent sterile dressings, and photographed on days 0, 3, 6, 9, 12, and 15 to calculate the affected surface areas using ImageJ software (Bethesda, MD) (Fig. 1).

2.8.2. Histologic examination

Healed skin samples on the 15th day of the affected mice were excised and fixed in 4 % paraformaldehyde (PFA), followed by

embedding in paraffin. Sections of 5 μm were immunohistochemically stained with anti-CD31 antibody, as described below; After blocking with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature, tissue sections were incubated with rabbit anti-CD31 mouse antibodies (1:100) (Abcam, Cambridge, United Kingdom), followed by incubation with secondary antibodies conjugated with peroxidase and stained with DAB to visualize antigens. Vessels composed of endothelial cells stained with an anti-CD31 antibody were observed under a microscope. A visual count of the stained vessels per high-power field (hpf) was performed in five randomly selected fields per sample, using light microscopy (BZ-X710; Keyence, Osaka, Japan) (Fig. 1).

2.9. Statistical analysis

The results were subjected to nonparametric analysis due to the small sample size. Statistical analyses were performed using Mann-Whitney and Kruskal-Wallis tests with the JMP statistical program (SAS Institute, Cary, NC). Data are presented as medians and interquartile ranges (IQRs), unless otherwise specified. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Age and BMI of SVF donors: age and BMI showed no significant differences among donors

Regarding age and BMI, the S-SVF group presented with median values of 50.5 (47.5–57.25) years and 21.85 (20.625–22.875) kg/m^2, respectively, while the L-SVF group had means of 53.5 (48.75–63.25) years and 23.05 (21.7–26.625) kg/m^2. Statistical analysis revealed no significant differences between the groups for either age or BMI ($P = 0.6291$ and $P = 0.146$, respectively) (Table 1).

3.2. Live and dead staining: Cell viability of SVF declined after long-term cryopreservation

For viability analysis, SVF cells were dyed in green and red colors for live and dead cells immediately after thawing. The rate of live cells in L-SVF was 62.11 (55.74–70.71) %, which was significantly lower than 75.33 (69.52–81.36) % in S-SVF ($P < 0.0001$) (Fig. 2).

3.3. Colony forming assay: proliferative potential of SVF cells was reduced after long-term cryopreservation

To assess proliferative potential, a colony forming assay was conducted. The number of colonies formed within a specific area in L-SVF group was 31.5 (25–49.25), which was significantly lower than the 58 (48–96) observed in S-SVF group ($P < 0.0001$) (Fig. 3).

3.4. Flow cytometry: phenotype of SVF cells declined after long-term cryopreservation

Cell surface markers of ADSCs expanded from SVF cells were phenotypically analyzed using flow cytometry. The expression of CD44 and CD90, markers indicative of mesenchymal stem cells, was assessed. The rate of CD44-positive cells in the S-SVF group was 95.8 % ± 2.8, which was significantly higher than the 90.2 % ± 5.6 observed in the L-SVF group ($P = 0.03$). Similarly, the rate of CD90-positive cells was 96.6 % ± 2.07 in S-SVF, significantly higher than 76.07 % ± 8.1 in L-SVF ($P = 0.005$) (Fig. 4).

3.5. Quantitative Real-Time PCR (qRT-PCR): gene expression of stem cell-specific markers in SVF cells was maintained after long-term cryopreservation

To assess gene expression, we quantified the relative mRNA levels of Nanog and Oct4 in ADSCs expanded from cryopreserved SVF cells. These genes are characteristically expressed in stem cells. The relative expression level of Nanog in the L-SVF group was approximately 0.40-fold higher than in the S-SVF group, where this difference was not statistically significant. ($P = 0.09$). Similarly, the relative expression level of Oct4 in the L-SVF group was approximately 0.40-fold higher than in the S-SVF group, where this difference was also not statistically significant ($P = 0.06$) (Fig. 5).

3.6. Differentiation: differentiation potential of SVF cells was partially reduced but maintained after long-term cryopreservation

We evaluated the multipotency of cryopreserved SVF cells by comparing the differentiation potential into adipogenic, osteogenic, and chondrogenic lineages between the S-SVF and L-SVF groups. Induced cells from these groups underwent specific staining to indicate differentiation: Oil Red O for adipocytes, Alizarin Red S for osteocytes, and Safranin O for chondrocytes. Subsequently, we quantified the relative mRNA levels of markers specific to these differentiated cells. The mRNA expression of FABP, a gene characteristic of adipocytes, was approximately 0.37-fold lower in the L-SVF group compared to the S-SVF group ($P = 0.012$). The mRNA expression of Osteopontin, a gene characteristic of osteocytes, was approximately 0.49-fold lower in the L-SVF group compared to the S-SVF group ($P = 0.036$). Additionally, the mRNA expression of Aggrecan, a gene characteristic of chondrocytes, was approximately 0.60-fold lower in the L-SVF group compared to the S-SVF group ($P = 0.17$). While the mRNA levels of FABP and Osteopontin were significantly higher in the S-SVF group, the difference in Aggrecan expression between the groups was not statistically significant (Fig. 6).

3.7. Wound-healing assessment: cryopreserved SVF cells promoted wound healing, but the rate of acceleration decreased with longer storage periods

We evaluated whether long-term preserved SVF cells maintained their ability to promote wound healing by comparing them with a short-term preservation group and a control group. We observed accelerated wound closure and enhanced angiogenesis attributed to the effects of cryopreserved SVF cells. Initially, we measured and compared the healed surface areas of wounds in mice between different groups. As depicted in Fig. 8, the healed areas in both the S-SVF and L-SVF groups were significantly larger than those in the control group from day three onwards ($P < 0.005$). However, in a direct comparison of S-SVF and L-SVF, there was no significant difference between the two groups until day 9, but after day 12, the wound healing effect of the L-SVF group significantly decreased (Fig. 7). Subsequently, we assessed and compared the number of blood vessels, which were immunostained using anti-CD31 antibodies, within the healed wounds. The counts per a high-power field were 22.5 (18.75–23.25) in the S-SVF group, 15 (13–17) in the L-SVF group, and 9 (7.75–10.25) in the control group. The number of vessels in both the S-SVF and L-SVF groups was significantly higher than that in the control group ($P < 0.001$ for both comparisons), and the vessel count in the S-SVF group was significantly greater than in the L-SVF group ($P < 0.001$) (Fig. 8).

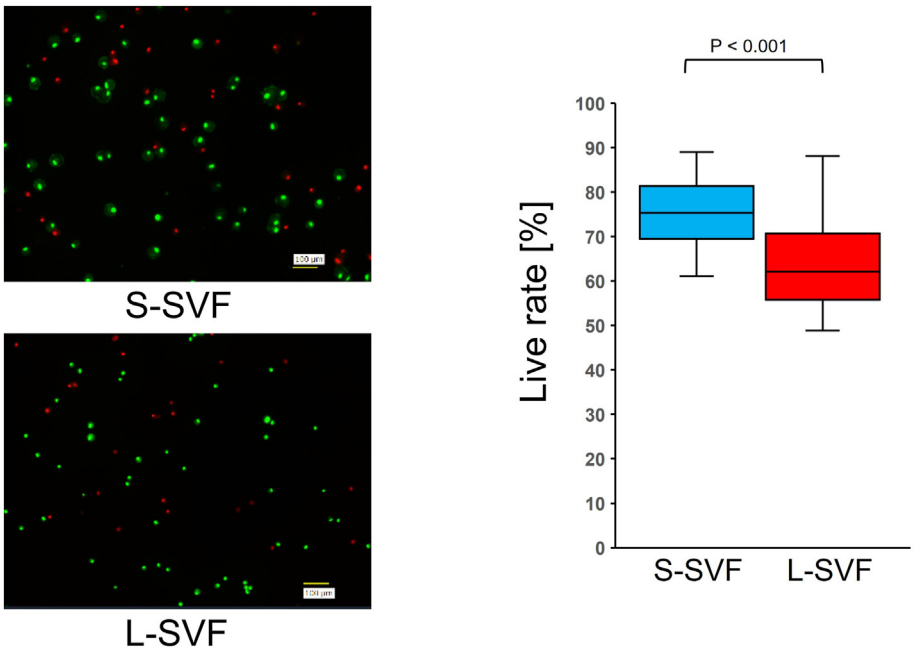

Fig. 2. Viability of SVF cells declined after long-term cryopreservation. (*Left*) Representative stained cells observed in a microscope, where green cells are live and reddish orange cells are dead. *Scale bar* = 100 μm. (*Right*) Quantitative data of live cells (n = 6 each). The proportion of live cells was significantly higher in S-SVF than in L-SVF. The data are expressed as medians with IQRs.

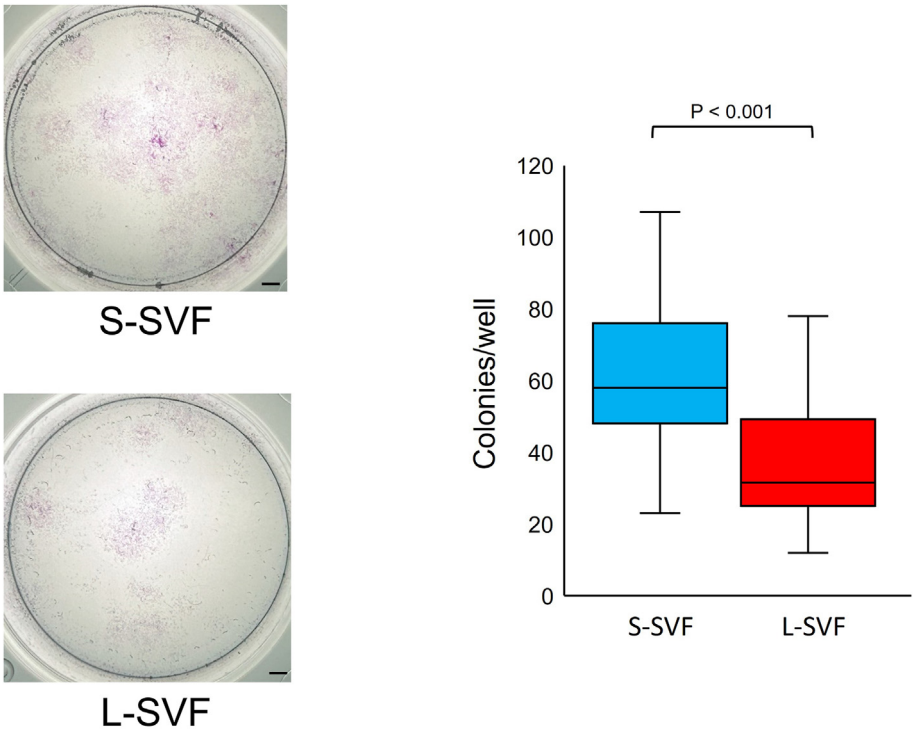

Fig. 3. Proliferative potential of SVF cells was reduced after long-term cryopreservation. (*Left*) Representative colonies observed in a macroscopic view. *Scale bar*=1000 μm. (*Right*) Comparison of the number of colonies between S- and L-SVF (n = 6 each). Formed colonies were significantly more in S-SVF than in L-SVF. The data are expressed as medians with IQRs.

4. Discussion

SVF is utilized as a readily available resource of ADSCs and is promising for regenerative therapies due to its wound-healing capacity [10,24]. Numerous reports have been published on the wound-healing ability of SVF cells, and many clinical trials are currently underway to utilize this function for the treatment of intractable diseases [8,25–28]. SVF cells promote tissue formation through paracrine signaling and interactions between various cell populations within the SVF and their host environment [29,30]. The

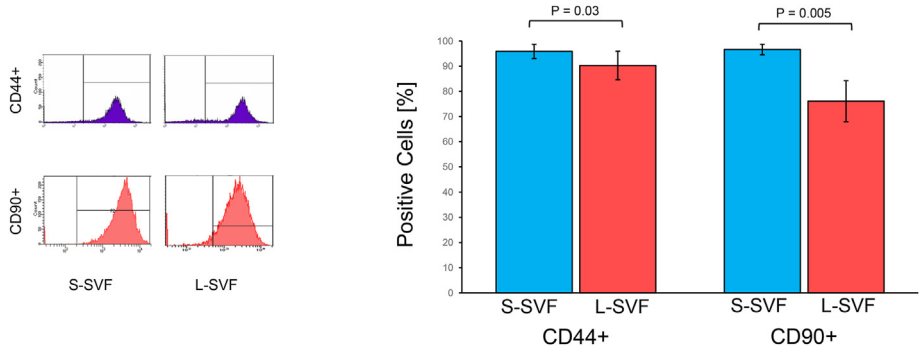

Fig. 4. Phenotype of SVF cells declined after long-term cryopreservation. (*Left*) Representative histograms of each marker in S-SVF and L-SVF groups. (*Right*) Comparison of the rate of CD44-and 90- positive cells between S- and L-SVF (n = 6 each). For both surface markers, the rate of positive cells was significantly higher in S-SVF than in L-SVF. The data are expressed as mean ± SD (Standard Deviation).

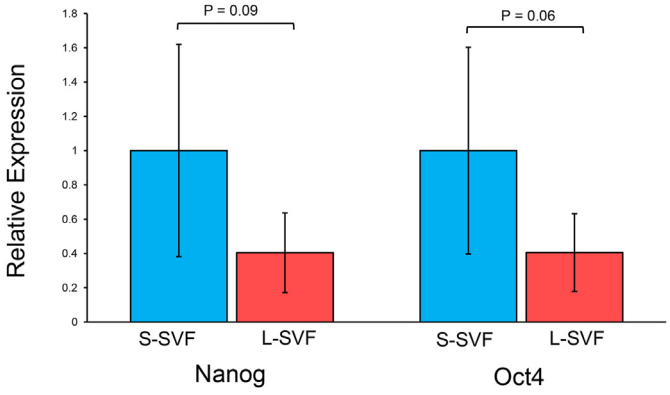

Fig. 5. Gene expression of stem cell-specific markers in SVF cells was maintained after long-term cryopreservation. (*Left*) Comparison of relative gene expression of Nanog between S- and L-SVF (n = 6 each). (*Right*) Comparison of relative gene expression of Oct4 between S- and L-SVF (n = 6 each). Both gene expressions did not differ significantly between S-SVF and L-SVF groups. The data are expressed as mean ± SD.

mechanism of wound healing facilitated by SVF cells relies on its heterogeneous composition, enabling functions such as anti-inflammation, angiogenesis, antioxidant effects, antifibrosis, and lymphatic vessel regeneration [28,29,31–38]. These capabilities are further supported by the secretion of extracellular matrix and the differentiation and proliferation of ADSCs into functionally specialized cells [10,39–41].

Despite its relatively safe clinical profile, liposuction remains an invasive procedure, and its repeated use may increase morbidity and potentially limit the clinical application of SVF cells. Additionally, it is known that the tissue regeneration capacity of SVF cells decreases with donor age [20,21]. Therefore, one solution to enable repeated administration of SVF cells without the need for repeated liposuction and to maintain the quality of the therapeutic product is cryopreservation. Cryopreserved cells are cost-effective and convenient to use. In particular, the potential to cryopreserve SVF from younger donors for later use in aging patients offers practical advantages for regenerative applications.

Cryopreserving SVF cells has been widely studied, with short-term storage (two weeks to six months) shown to generally maintain cell surface markers, proliferation, and differentiation potential after thawing, although viability varies among reports [42–47]. Feng et al. have reported that one-month cryopreserved SVF cells maintained tissue integrity and cell viability, resulting in a better long-term retention rate than that of cryopreserved fat, as well as showed angiogenic capacity in the accelerated healing rate of ischemic wounds in vivo assays [48]. Furthermore, Kamenaga et al. [49] have reported that short-term cryopreserved SVF cells possessed an equivalent fracture healing capacity to fresh SVF by promoting angiogenesis and osteogenesis in vivo assay.

In contrast, research on long-term cryopreservation is more limited. Kokai et al. [50] reported on the cellular analysis of human SVF cells cryopreserved for 7–12 years. They demonstrated that cell surface markers, proliferation capacity, and differentiation potential of ASDCs isolated and cultured from cryopreserved SVF cells did not significantly change. However, the preservation method for this SVF cells was confidential, and crucial assessments of cell function were not conducted. Subsequently, Kumar et al. [51] reported that ADSCs cryopreserved for 12 years in a storage solution containing

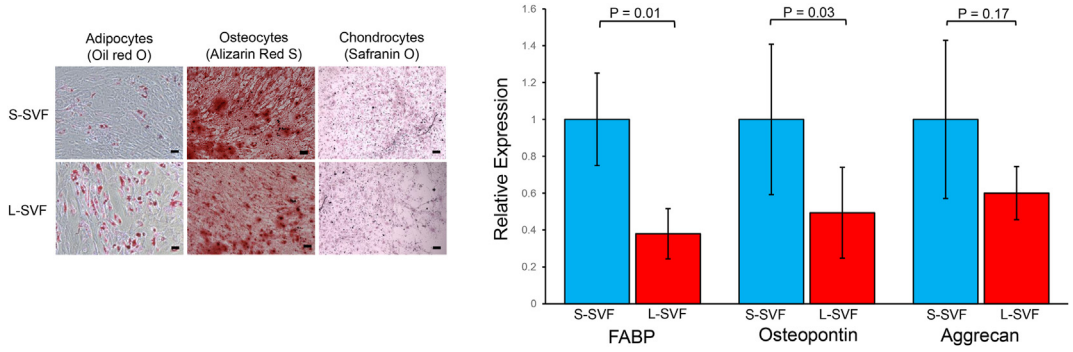

Fig. 6. Differentiation potential of SVF cells was partially reduced but maintained after long-term cryopreservation. (*Left*) Representative images of three cell types induced from S- and L-SVF cells (adipocytes, osteocytes, and chondrocytes), each specifically stained. *Scale bar* = 100 μm. (*Magnification* = ×400). (*Right*) Comparison of mRNA levels of the three types of induced cells between S- and L-SVF (n = 6 each). The mRNA levels of FABP and Osteopontin were higher in S-SVF, whereas Aggrecan was not significantly different between both groups. The data are expressed as mean ± SD.

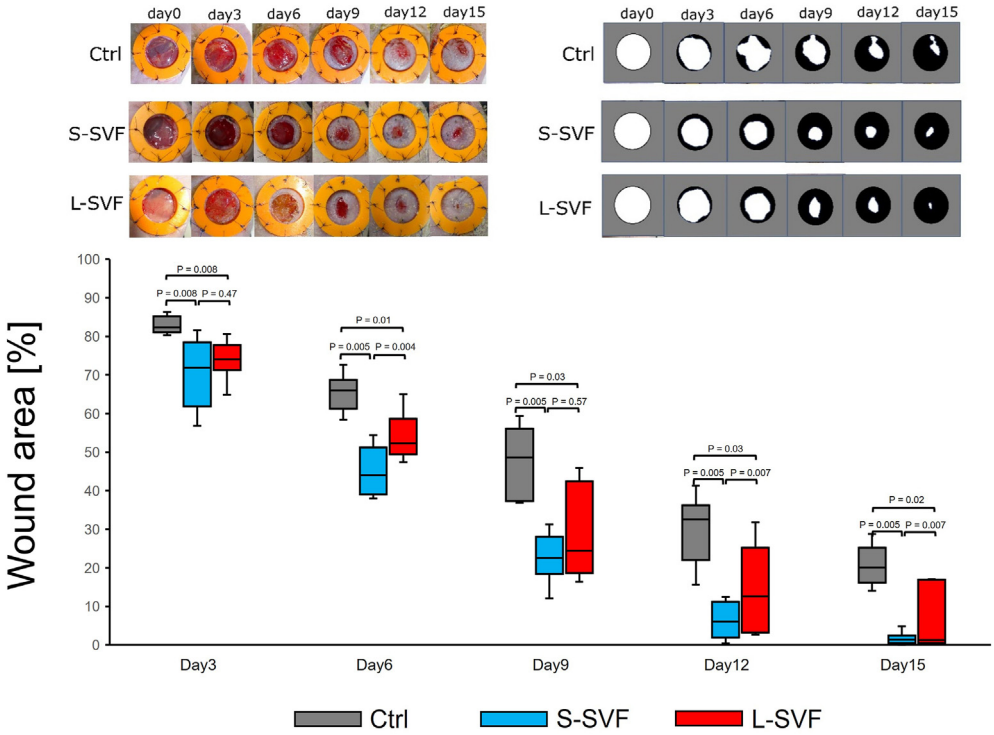

Fig. 7. Cryopreserved SVF cells promoted wound healing, but the rate of acceleration decreased with longer storage periods. (*Above*) Representative images of wound healing in each group. The wound sizes on days 0, 3, 6, 9, 12, and 15 were measured on digital photographs using ImageJ software. (*Below*) Quantitative evaluation of wound areas of the three groups on each day (n = 6 each). Wound healing was accelerated in the S- and L-SVF groups more than in the control group, while in the S-SVF group more than in the L-SVF group. The data are expressed as medians with IQRs.

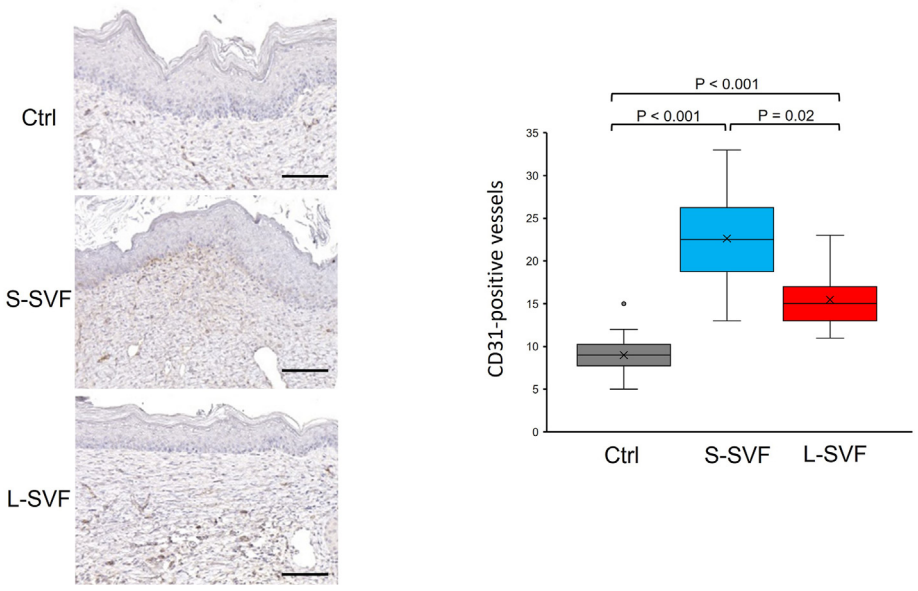

Fig. 8. Cryopreserved SVF cells enhanced angiogenesis, but its efficacy declined with extended freezing durations. (*Left*) Representative microdissections immunostained with anti-CD31 antibodies, indicating neovascularization. Vessels are stained with DAB for anti-CD31 antibody, as a brown color. *Scale bar* = 100 μm. (*Right*) Comparison of the number of stained vessels in a high-power field of the healed tissues (n = 6 each). The number of vessels was greater in the S- and L-SVF groups than in the control group, while in the S-SVF group was greater than in the L-SVF group. The data are expressed as medians with IQRs.

20 % FBS and 10 % DMSO retained nearly all cell characteristics, and suggested potential wound healing effects in vitro using assays such as the trabecular meshwork cell wound healing assay and scratch assay. However, the study focused on ADSCs and did not examine in vivo wound healing capabilities. On the other hand, Shaik et al. [52] reported that the use of cryoprotectants similar to those in our study resulted in a decline in osteogenic differentiation potential after a 10-year period of cryopreservation, which was consistent with our findings. Research findings on cell characteristics and functions, including stem cell markers, after cryopreservation of human SVF cells or ADSCs are summarized in Table 2 [42–55].

Table 2
The summary of research findings on cell characteristics and functions, including stem cell markers, after cryopreservation of human SVF cells or ADSCs.

NO.	Authors	Publish year	Title of manuscript	Nations	Journal	SVF or ADSCs	Animal	Cryoprotective agents	Freeze period	Stemness analysis			Functional analysis		
										Parameters	Methods	Results	Parameters	Methods	Results
1	Gonda K, Shigeura T, Sato T	2008	Preserved proliferative capacity and multipotency of human adipose-derived stem cells after long-term cryopreservation [42]	Japan	Plast Reconstr Surg	ADSCs	Human	Cell Banker 1 (Wako Chemicals co., Ltd., Osaka, Japan) (The manufacturer does not disclose its ingredients but states that it contains fetal bovine serum.)	6 months	Proliferative capacity, multipotency and cell surface markers in vitro	Doubling time, differentiation assays and flow cytometry	No significant difference with fresh SVF	–	–	–
2	Thirumala S, Gimble JM, Devireddy RV	2010	Cryopreservation of stromal vascular fraction of adipose tissue in a serum-free freezing medium [45]	USA	J tissue Eng Regen med	SVF	Human	DMEM with 10 % PVP (polyvinylpyrrolidone) or DMEM with serum and DMSO	2 weeks	Differentiation assay in vitro	Immunostaining	Maintained	–	–	–
3	Minozio G, Corazza M, Mariotta L	2014	Frozen adipose-derived mesenchymal stem cells maintain high capability to grow and differentiate [43]	Switzerland	Cryobiology	SVF	Human	5 % human albumin solution with 5 % DMSO	14–193 days	Differentiation assay in vitro	Immunostaining	Maintained	–	–	–
4	Jang S, Yoon W, Kook K	2016	Long term cryopreservation of stromal vascular Fraction (SVF) and isolation of SVF from cryopreservation fat: A preclinical application [53]	Korea	Cytotherapy	SVF	Human	Undescribed	8 weeks	Cell viability in vitro	Cell count	Reduction to 40 %	–	–	–
5	Kokai LE, Traktuev DO, Zhang L	2017	Adipose stem Cell function maintained with age: An Intra-Subject study of long-term cryopreserved cells [50]	USA	Aesthet Surg J	SVF	Human	Confidential	7–12 years	Cell surface markers, proliferation and differentiation assay in vitro	Flow cytometry, cell population doubling time and immunostaining/ RT-PCR	Significant differences in SVF subpopulations and ADSCs function are patient specific and do not appear to change much after aging	–	–	–
6	Agostini F, Rossi FM, Aldinucci D	2018	Improved GMP compliant approach to manipulate lipoaspirates, to cryopreserve stromal vascular fraction, and to expand adipose stem cells in xeno-free media [44]	Italy	Stem Cell res ther	SVF	Human	Low (5 %) DMSO concentration in pure serum	2 months	Cell surface markers, proliferation and differentiation assay in vitro	Flow cytometry, colony forming assay and differentiation assay	Minimally affected	–	–	–
7	Shaik S, Wu X, Gimble J	2018	Effects of Decade long freezing storage on adipose derived stem cells functionality [52]	USA	Sci Rep	ADSCs	Human	10 % DMSO (V/V) in FBS solution	10 years	Cell surface marker and differentiation in vitro	Flow cytometry and immunostaining/ RT-PCR	Above 95 % of stromal marker, a decrease of Osteopontin/ unchanged of adipogenic	–	–	–
8	Zanata F, Bowles A, Frazier T	2018	Effect of cryopreservation on human adipose tissue and isolated stromal vascular fraction cells: In Vitro and In vivo analyses [47]	Brazil	Plast Reconstr Surg	SVF	Human	80 % bovine calf serum, 10 % DMSO, and 10 % stromal medium	4–6 weeks	Cell proliferation and surface markers in vitro	Colony-forming assay and immunophenotype	Retained adhesive and proliferative properties and increased expression of stromal and adipogenic markers	–	–	–
9	Solodeev I, Orgil M, Bordeynik-Cohen M	2019	Cryopreservation of stromal vascular fraction cells reduces their counts but not their stem Cell potency [46]	Israel	Plast Reconstr Surg Glob Open	SVF	Human	90 % FBS and 10 % DMSO	6–8 weeks	Proliferation, surface markers and differentiation assay in vitro	Colony-forming assay, flow cytometry and immunostaining/ optical density	No significant difference with fresh SVF	–	–	–

(*continued on next page*)

Table 2 (*continued*)

NO.	Authors	Publish year	Title of manuscript	Nations	Journal	SVF or ADSCs	Animal	Cryoprotective agents	Freeze period	Stemness analysis			Functional analysis		
										Parameters	Methods	Results	Parameters	Methods	Results
10	Zheng W, Shen J, Wang H	2019	Effects of frozen stromal vascular fraction on the survival of cryopreserved fat tissue [54]	China	Aesthetic Plast Surg	SVF	Human	A cryoprotective agent (details unknown)	3 months	Adhesive and proliferative properties in vitro	MTT assay and morphologic assessment	Maintained	Survival of fat grafts in vivo (mice)	Weight, volume and morphology, and histochemical staining and immunohistochemistry	Increased survival of fat grafts
11	Kumar A, Xu Y, Yang E	2019	Fidelity of long-term cryopreserved adipose-derived stem cells for differentiation into cells of ocular and other lineages [51]	USA	Exp Eye res	ADSCs	Human	70 % DMEM/HAMs F12, 20 % FBS and 10 % DMSO	12 years	Cell surface marker, qRT-PCR, proliferation and differentiation assay in vitro	Flow cytometry, qRT-PCR, proliferation, colony-forming assay and immunostaining	Maintained	Trabecular meshwork cell wound healing assay in vitro	Scratch assay, gene expression of fibrosis	Promoted wound healing of TM cells, reduced fibrosis
12	Feng J, Hu W, Fanai J	2019	Mechanical process prior to cryopreservation of lipoaspirates maintains extracellular matrix integrity and cell viability: Evaluation of the retention and regenerative potential of cryopreserved fat-derived product after fat grafting [48]	China	Stem Cell res ther	SVF	Human	Without cryoprotectant	1–3 months	Tissue viability, ADSC function, and the extracellular content in vitro	Morphologic assessment, colony-forming assay, differentiation assay and Western blot analysis	Preserved original shapes and ECM. Significant lower colonies than fresh SVF. Maintained differentiation potential.	Fat grafting and ischemic wound healing model in vivo (mice)	Flow cytometry, history and immunofluorescence staining	Better long-term retention rate of fat grafts and decreased inflammatory cell infiltration and showed superior adipose tissue structure and accelerated ischemic wound healing process
13	Shaik S, Wu X, Gimble J	2020	Non-toxic freezing media to retain the stem cell reserves in adipose tissues [55]	USA	Cryobiology	SVF	Human	DMSO, bovine serum albumin, lipoaspirate saline, human serum albumin and Polyvinylpyrrolidone	1 month	Cell surface markers, proliferation and differentiation assay in vitro	Flow cytometry, immunostaining and qRT-PCR	Maintained	–	–	–
14	Kamenaga T, Kuroda Y, Nagai K	2021	Cryopreserved human adipose-derived stromal vascular fraction maintains fracture healing potential via angiogenesis and osteogenesis in an immunodeficient rat model [49]	Japan	Stem Cell res ther	SVF	Human	20 % human serum albumin and 10 % DMSO	3 months	Angiogenic and osteogenic differentiation in vitro	Immunostaining, tube length, ALP activity and mineralization	No significant difference with fresh SVF	Fracture healing in vivo (mice)	Radiologic, histological, immunohistochemical and biomechanical analyses	No significant difference with fresh SVF

Our study on the long-term storage of SVF involved analyzing the cell characteristics of SVF samples cryopreserved for over 12 years, including the longest duration of 13 years, from six patients. We compared these with SVF samples from six patients that had been cryopreserved for a short term, for which stem cell and wound healing capabilities had already been proven to be maintained. Our results showed a significant decrease in cell survival from 75 % to 62 % with extended storage beyond 10 years, indicating that a substantial proportion of cells, exceeding half, remained viable. However, proliferative capacity decreased to about half after a 10-year storage period, and expression of ADSCs markers and differentiation potential also significantly declined. For instance, we observed non-significant differences in Nanog and Oct4 expression, which may be clinically relevant despite the lack of statistical significance. High inter-individual variability observed in the short-term SVF storage group, consistent with previous observations by Kumar et al. [51], could contribute to these findings.

To our knowledge, no studies have confirmed the therapeutic effects of long-term (up to 13 years) storage of SVF cells on wound healing. We created a wound healing model in nude mice to evaluate the healing potential of SVF cells after long-term cryopreservation, where more than half of the cells remained viable. Our findings indicated that while L-SVF exhibited somewhat reduced wound healing effects compared to S-SVF, though it remained more effective than the control group at all measured time points. This was further supported by similar trends observed in the assessment of wound neovascularization. Nevertheless, it can also be interpreted that both stem cell potential and wound healing capability were clearly diminished due to prolonged cryopreservation. These reductions in viability and stemness could be attributed to factors such as ice crystal formation, osmotic stress, oxidative damage, or even epigenetic changes occurring during long-term storage [56,57]. Such mechanisms possibly lead to the reduction in gene and protein expression levels in SVF cells. Particularly, we hypothesize that the decreased expression of cytokines involved in angiogenesis, such as VEGF, HGF and Adiponectin, serves as critical indicators of the impacts of long-term cryopreservation [58–62]. Long-term cryopreservation may also lead to instability in the expression of genes involved in maintaining stemness, such as LNGFR and VCAM-1 [63]. The alterations in genetic stability and secretory factor profiles could explain the outcomes observed in our study, which demonstrated a decline in stemness and angiogenic response in wound healing. To further validate these findings, future studies should quantify these cytokines and gene expression, as well as employ neutralizing antibody assays and siRNA-mediated knockdown experiments. Measuring the potency of these effects would be invaluable for assessing the functionality and quality of cryopreserved cells before their clinical application.

Cryopreservation is an indispensable technique widely used for the preservation of cells intended for cell biology research and cell therapy. Its applications are diverse, including the cryopreservation of various cell lines for cell banks, and sperm, oocytes, and embryos for livestock production, and reproductive cells for reproductive medicine [64]. There is also widespread discussion regarding the banking of human iPS cells for clinical applications in regenerative medicine [65,66]. As new cryopreservation technologies emerge, innovations like rapid cooling, DMSO-free cryoprotectants, and controlled-rate freezing may help preserve cell viability and functionality over extended periods. Exploring these options in future studies could further optimize SVF storage.

Maintaining cell viability during freezing and thawing presents various challenges, with the most notable being the formation of ice crystals both intracellularly and extracellularly. SVF cells comprise mesenchymal stem cells, adipose progenitor cells, endothelial progenitor cells, pericytes, immune cells, hematopoietic cells, and other stromal components [9]. Given the heterogeneous nature of SVF, each cell type is affected differently by freezing [45,46,54]. Hematopoietic cells, in particular, are more susceptible to the freezing process, possibly resulting in affected biological activity of SVF cells [47].

Our study has demonstrated that even with basic, classical freezing methods, SVF cells preserved for over 12 years retains sufficient performance to be used as a clinical cell therapy material. Nonetheless, it is essential to consider the variability introduced by using different donors for the short-term and long-term groups, as this may impact the study's findings. Future studies could address this by using same-donor comparisons over varying cryopreservation durations to better control for cross-donor variability. Advances such as the use of controlled-rate freezers, rapid long-term cryopreservation using −80 °C deep freezers, and the development of new DMSO-free cell freezing media are ongoing. These innovations aim to ensure that the stem cell potential and tissue regenerative support functions of cells remain intact even after extended periods of cryopreservation.

There are some limitations to our current study, including the small number of animals used in the experiments and the variability in cryoprotective agents reported in the literature. Future research could explore more advanced techniques and alternative cryoprotectants, with a focus on optimizing long-term cell viability and minimizing functional losses.

5. Conclusion and future directions

SVF cells that have been cryopreserved for more than 10 years using classic liquid nitrogen show reductions in viability, stem cell potential, and wound healing ability over the long preservation period. However, these abilities do not decrease by half, and certain functional aspects remain comparable to those in the short-term cryopreservation group. This suggests that SVF retains a degree of regenerative potential even after extended storage, highlighting the feasibility of long-term cell banking for clinical applications. However, it remains unclear whether these cryopreserved cells are economically viable and useful in actual clinical practice when considering aspects such as cell preservation.

Future research should focus on developing optimized cryopreservation protocols to further enhance the functionality of SVF during long-term storage. Additionally, exploring alternative cryoprotectants and advanced techniques, such as controlled-rate freezing, may help maintain cell viability and stemness. Specific studies should also assess the regenerative capabilities of mid-aged long-term cryopreserved SVF compared to freshly prepared SVF from older donors. Examining these factors will be essential for advancing SVF applications in regenerative medicine, particularly for aging patients and those requiring personalized cell banking solutions.

Author contributions

Conceptualization, N.I., and Yo.So.; methodology, Yo.So.; investigation, N.I., Yo.So., T.K., and S.S.; supervision, Yo.So., T.N., and O.M.; original draft preparation, N.I., and Yo.So.; writing-review and editing, Yo.So., E. H. N., T.N., K.Y., Yu.Sh., and O.M.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve language and readability. After using this tool/

service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2025.02.004.

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