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ORIGINAL RESEARCH

COMPARISON OF STRUCTURAL INTEGRITY AND VIABILITY OF SPLIT- THICKNESS GRAFTS STORED AT 4℃ AND -8℃

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ABSTRACT

Background: Skin grafting is a widely utilized modality for wound closure. However, suitable donor grafts are not always readily available, and in some instances the wound bed is not yet prepared for graft application. Consequently, the ability to preserve split-thickness grafts (STG) is essential in reconstructive practice.

Objective: This study aims to compare the structural integrity and viability of split-thickness grafts stored at 4°C and -80°C.

Methodology: This experimental study employed a post-test control group design, utilizing 21 split-thickness skin samples obtained from excess skin during surgical procedures at Dr. Soetomo Regional Hospital, Surabaya. Samples were divided into three groups: fresh (control), storage at 4°C, and storage at -80°C for two weeks using 0.9% NaCl and glycerol as storage media. Tissue structure was assessed via hematoxylin-eosin staining, while cell viability was measured using the MTT Assay.

Results: Results demonstrated that storage at -80°C more effectively preserved the integrity of the epidermal and dermal structures, particularly concerning de-keratinization and the dermo- epidermal junction, compared to storage at 4°C ($p < 0.05$). Tissue viability increased with decreasing storage temperature, reaching the highest values at -80°C for both 0.9% NaCl and glycerol media ($p < 0.001$). The use of glycerol as a storage medium yielded higher viability compared to 0.9% NaCl. Lowering the storage temperature significantly enhanced cell survival and maintained tissue architecture.

Conclusion: Storing split-thickness grafts at -80°C in glycerol provides the optimal preservation of tissue structure and viability, representing an effective long-term storage method for use in plastic surgery therapies.

Keywords: split-thickness graft, skin storage, cryopreservation, skin structure, skin viability

INTRODUCTION

Skin grafting is one of the most frequently used wound-closure techniques in plastic surgery and has been practiced for more than 2,500 years. Beyond its cosmetic advantages, skin grafting restores skin continuity, providing essential protective

function to the body. However, donor skin grafts are not always available in adequate amounts throughout the course of treatment, and in some cases the wound bed is not yet ready to receive a graft. Clinicians may occasionally harvest more skin than immediately required so that excess tissue temperature of the grafts (4°C and −80°C), while the dependent variables were structural characteristics and tissue viability.

Study Subjects and Sample Selection

The study subjects consisted of split- thickness skin grafts obtained from excess tissue removed during surgical procedures, including abdominoplasty, blepharoplasty, skin grafting, and facelift surgeries. The study population included all split-thickness grafts harvested at Dr. Soetomo General Hospital, Surabaya. Eligible samples were obtained from patients aged 30–60 years who provided informed consent. Exclusion criteria included crush injury, the presence of striae/stretch marks, and a history or high risk of AIDS, hepatitis, or syphilis.

Sample size determination referred to Knapik (2012), who utilized 17 samples in a similar study. Considering

the availability of cases, this study employed 21 samples, which were distributed equally into three groups.

A split-thickness skin graft was defined as a graft containing the full thickness of the epidermis and part or all of the dermis, depending on the desired thickness (Perdanakusuma, 2005).

Graft Harvesting and Initial Processing

Following informed consent, eight split-thickness grafts measuring 20 × 10 mm with a thickness of 0.30–0.45 mm were harvested using a Humby knife under general anesthesia. Grafts were placed in sterile containers containing 0.9% NaCl supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin and transported at 4°C. Samples were washed sequentially in povidone-iodine, chlorhexidine digluconate, and isopropanol.

Experimental Groups and Storage Procedures Grafts were allocated into three groups:

a. Fresh group (control): Underwent immediate microscopic examination at the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Airlangga, and MTT assay evaluation at Gamma Scientific Biolab, Malang.

b. 4°C storage group: Grafts were immersed in a solution containing streptomycin sulfate and sodium penicillin G, wrapped in saline- moistened gauze, and stored at 4°C for two weeks.

c. −80°C storage group (glycerolized grafts):

Grafts underwent a three-stage glycerolization protocol:

• Immersion in 98% glycerol + 0.9% NaCl (50/50 v/v) with antibiotics, followed by visual inspection for mechanical damage.

• Placement in sterile plastic with 98% glycerol and 0.9% NaCl (10/14 v/v; final concentration 70% glycerol), incubated at 33°C for 3 hours with rotational shaking, followed by reevaluation.

• Placement in sterile plastic with 85% glycerol, incubation at 33°C for 3 hours, then trimming, cutting, and packing in 85% glycerol.

The processed grafts were stored at −80°C for two weeks.

Histological Assessment

After two weeks, each graft was bisected. One portion was processed for microscopic evaluation using hematoxylin– eosin staining. Structural parameters assessed included epidermal de-keratinization, epidermal integrity, ballooning degeneration of epithelial cells, dermo-epidermal junction morphology, dermal gland appearance, and vascular structure. Each parameter was scored from 0 (none) to 3 (severe), based on the proportion of tissue affected.

Tissue Viability Assessment (MTT Assay)

The second portion of each graft was used for viability testing using the MTT assay. Keratinocytes isolated from the grafts were exposed to MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H- tetrazolium bromide). This reagent permeates viable cell membranes and mitochondria, where enzymatic reduction yields MTT- formazan crystals. Optical density (OD), measured at approximately 570 nm, reflects intracellular formazan concentration and serves as an indicator of cellular metabolic activity and viability (Ghasemi, 2021).

Study Sites and Timeline

The study was conducted at the Anatomical Pathology Laboratory of the Faculty of Medicine, Universitas Airlangga, Surabaya, and at Gamma Scientific Biolab, Malang. The overall timeline—from protocol preparation, laboratory work, data analysis, to manuscript completion—spanned approximately nine months.

MATERIALS AND EQUIPMENT

Materials and equipment included instruments for harvesting split-thickness grafts, refrigeration and cryogenic storage units (4°C and −80°C), glycerol solutions, antibiotic preparations, microtomy and staining equipment, and all reagents, media, and enzymes required for the MTT assay.

Data Processing and Analysis

The collected data were tabulated, followed by tests of normality and homogeneity of variance to determine whether the data were normally distributed and homogeneous. If the results indicated a normal distribution, the analysis was continued using parametric statistical methods, specifically analysis of variance (ANOVA) with a 95% confidence level. When ANOVA demonstrated a statistically significant difference, post-hoc analysis was performed using the Least Significant Difference (LSD) test.

RESULTS

A total of 21 split-thickness skin graft samples were analyzed and divided into three groups: fresh (control), 4°C, and −80°C (each n = 7). Evaluation included the structure of both epidermis and dermis. Epidermal parameters assessed were de-keratinization (DK), epidermal integrity (EI), ballooning of the epithelial cells (BL), and the dermo- epidermal junction (DEJ). Dermal structure was evaluated based on dermal gland appearance (DGL) and blood vessel structure (BV) (Table 1.).

Table 1. Descriptive and Comparative Test Results for Histological Examination

Histological Parameter	Group	None (%)	Mild (%)	Moderate (%)	Severe (%)	p-Value
De-keratinization (DK)	Control	0.0	0.0	0.0	100.0	0.002
	4°C	28.6	42.9	28.6	0.0	
	–80°C	14.3	14.3	28.6	42.9	
Epidermal Integrity (EI)	Control	57.1	42.9	0.0	0.0	0.172
	4°C	57.1	42.9	0.0	0.0	
	–80°C	28.6	28.6	28.6	14.3	
Ballooning of Epithelial Cells (BL)	Control	57.1	28.6	14.3	–	0.762
	4°C	42.9	28.6	28.6	–	
	–80°C	57.1	28.6	14.3	–	
Dermo-epidermal Junction (DEJ)	Control	28.6	71.4	0.0	0.0	**0.001**
	4°C	0.0	0.0	28.6	71.4	
	–80°C	42.9	42.9	14.3	0.0	
Dermal Glands Appearance (DGL)	Control	0.0	14.3	57.1	28.6	0.413
	4°C	28.6	57.1	14.3	0.0	
	–80°C	28.6	57.1	14.3	0.0	
Blood Vessels Structure (BV)	Control	0.0	14.3	57.1	28.6	0.135
	4°C	28.6	57.1	14.3	0.0	
	–80°C	28.6	57.1	14.3	0.0	

The control group demonstrated the most severe tissue damage across all parameters, particularly DK, with 100% of samples showing severe damage. Storage at 4°C showed predominantly mild damage, whereas storage at −80°C resulted in the highest preservation of tissue integrity, especially in DK and DEJ. EI remained relatively stable in both the control and 4°C groups; however, grafts stored at −80°C showed a greater proportion of mild damage. BL did not exhibit meaningful changes across temperature variations.

Histological results using glycerol storage media were consistent with those stored in 0.9% NaCl, demonstrating better structural preservation at lower temperatures. Most samples stored at −80°C exhibited no detectable DEJ damage, indicating that freezing at −80°C provided superior protection for the dermo-epidermal junction compared with storage at 4°C.

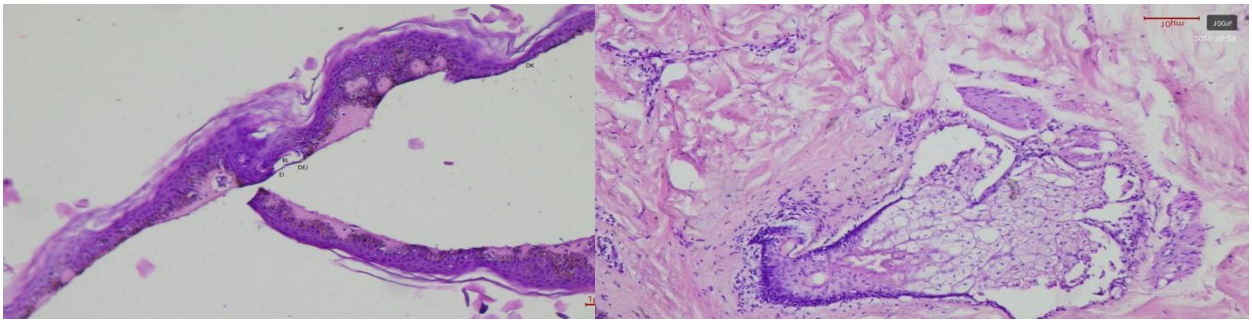

Figure 1. Fresh Split-Thickness Skin graft

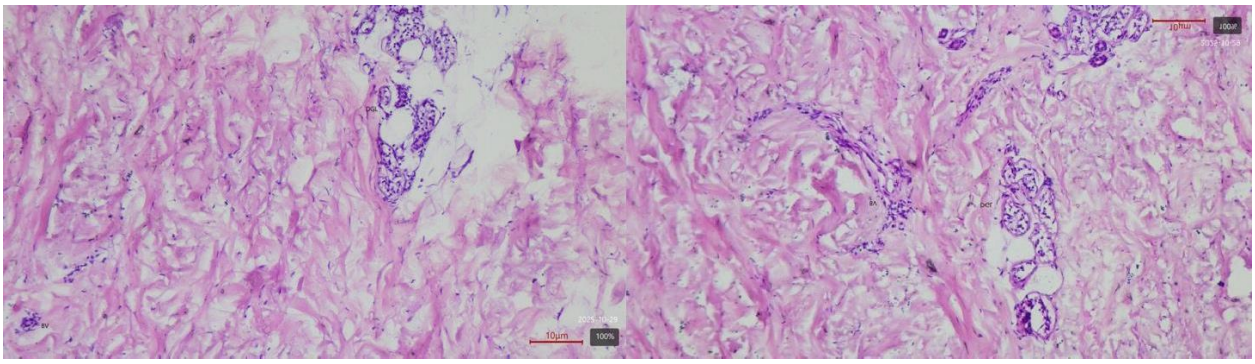

Figure 2. Split-Thick ness Skin Graft in NaCl 4°C

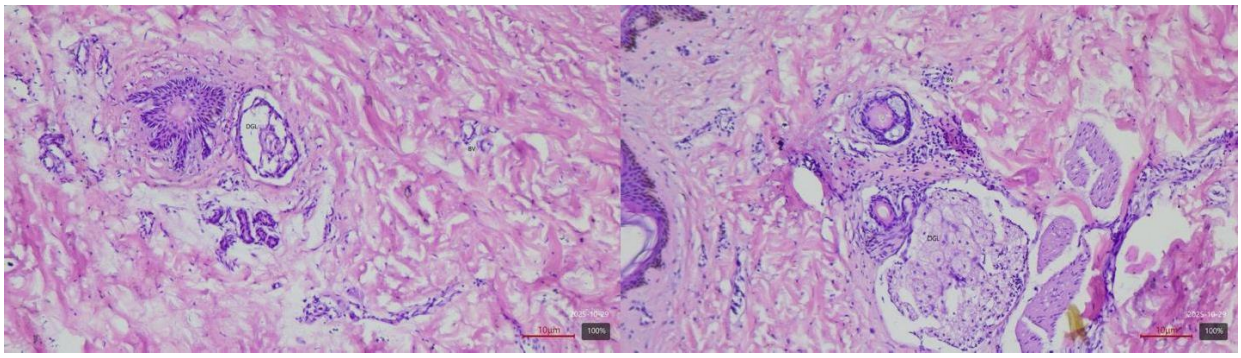

Figure 3. Split Thickness Skin Graft in NaCl −80°C

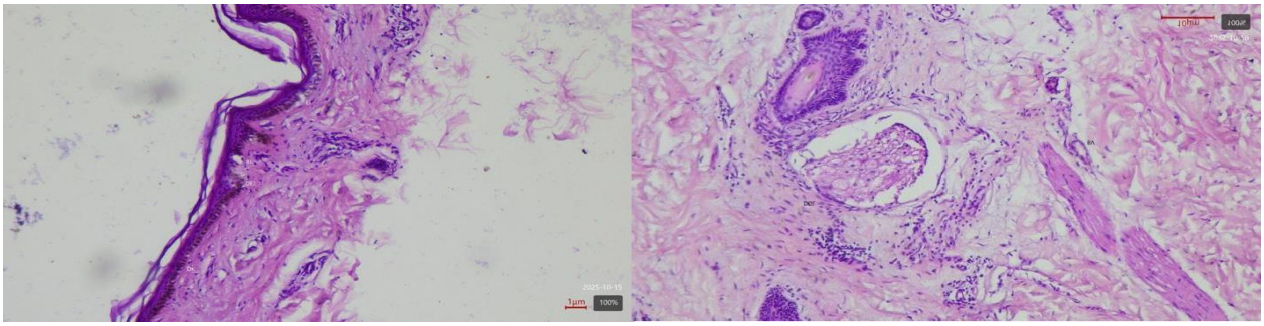

Figure 4. Split-Thickness Skin Graft in Glycerol 4°C

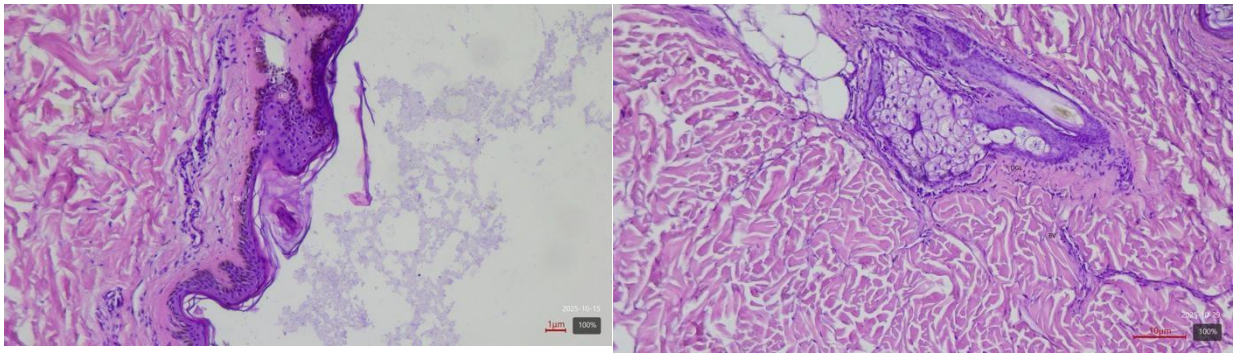

Figure 5. Split-Thickness Skin Graft in Glycerol −80°C

A Kruskal–Wallis test was performed to assess differences in histological structure among the treatment groups (control, 4°C,

−80°C) using 0.9% NaCl media. Significant differences were observed in DK and DEJ, with p-values of 0.002 and 0.001, respectively ($p < 0.05$). These findings indicate that storage temperature significantly affected tissue integrity in these layers.

EI and BL did not differ significantly between groups ($p = 0.172$ and $p = 0.762$), suggesting that temperature variations between 4°C and −80°C did not substantially influence these parameters. Overall, the results indicate that decreasing storage

dermo-epidermal junction.

In general, the distribution of damage severity showed that the control group had the highest proportion of moderate–severe damage in DGL and BV. Storage at 4°C and
−80°C decreased the proportion of moderate– severe damage and increased the "none" and "mild" categories, demonstrating a protective effect of lower temperatures on dermal structure. The most consistent findings were seen in samples stored with glycerol media, especially for BV, where samples stored at 4°C showed 100% none–mild damage with no moderate or severe damage. This pattern supports the statistical findings indicating that low-temperature storage—particularly at 4°C and −80°C— preserved dermal histology, including vascular structures. Glycerol storage media appeared more sensitive in detecting structural variability among groups.

Analysis of samples stored in glycerol demonstrated patterns consistent with those stored in 0.9% NaCl. Significant differences were observed in DK ($p = 0.001$) and DEJ ($p = 0.011$), while EI ($p = 0.062$) and BL ($p = 0.195$) remained statistically insignificant. These findings reinforce that the dermis and dermo-epidermal junction were the most temperature-sensitive components, with superior preservation at −80°C compared with 4°C or fresh controls.

Tissue Viability Study

In the 0.9% NaCl storage group, quantitative viability assessment revealed clear differences among treatment groups. Mean tissue viability increased with decreasing storage temperature: 50.47 ± 3.78% in the control group, 64.41 ± 2.35% at 4°C, and 74.04 ± 3.85% at −80°C. The low variability across samples indicates high result consistency. A similar pattern was observed in the glycerol storage group, though with higher viability at all temperatures: 50.47 ± 3.78% (control), 73.51 ± 4.40% (4°C), and 84.27 ± 5.03% (−80°C) (Table 2.).

Table 2. Tissue Viability Comparative Test Results

Storage Medium	Group	Mean ± SD	p-value
0.9% NaCl	Control	50.47 ± 3.78	<0.001
	4°C	64.41 ± 2.35	
	−80°C	74.04 ± 3.85	
Glycerol	Control	50.47 ± 3.78	<0.001
	4°C	73.51 ± 4.40	
	−80°C	84.27 ± 5.03	

ANOVA revealed highly significant differences across the three groups for both storage media ($p < 0.001$), indicating that storage temperature had a strong effect on tissue viability. Descriptively, these results show that lower storage temperatures corresponded with higher tissue viability.

The magnitude of group differences also suggests that glycerol had greater sensitivity in detecting viable cells, particularly in frozen tissues. These findings support the hypothesis that storage at −80°C better preserves tissue structure and function with minimal morphological and cellular degradation.

Post Hoc Analysis (LSD Test)

Post hoc analysis confirmed significant differences among all storage conditions, for both 0.9% NaCl and glycerol media, with $p < 0.001$ for all comparisons.

In the 0.9% NaCl group, mean viability increased significantly from 50.47% (control) to 64.41% (4°C) and 74.04% (−80°C). The differences between groups were consistent:

- Control vs. 4°C: +13.94%
- Control vs. −80°C: +23.57%
- 4°C vs. −80°C: +9.63%

These results indicate that each decrease in storage temperature corresponded to a significant increase in tissue viability.

A similar pattern occurred with glycerol, though with larger absolute differences. Mean viability increased from 50.47% (control) to 73.51% (4°C) and 84.27% (−80°C). The inter-group differences were:

- Control vs. 4°C: +23.04%
- Control vs. −80°C: +33.79%
- 4°C vs. −80°C: +10.76%

Overall, storage at −80°C produced the highest level of tissue viability. Additionally, glycerol consistently yielded higher viability values than 0.9% NaCl at all temperatures.

Table 3. Post Hoc (LSD) Test Results for Tissue Viability Based on Storage Temperature

Storage Medium	Group Comparison	Std. Error	p-value	Description
0.9% NaCl	Control – 4°C	1.82	<0.001	Significant
	Control – −80°C	1.82	<0.001	Significant
	4°C – −80°C	1.82	<0.001	Significant
Glycerol	Control – 4°C	2.37	<0.001	Significant
	Control – −80°C	2.37	<0.001	Significant
	4°C – −80°C	2.37	<0.001	Significant

DISCUSSION

Differences in Histological Structure of Split-Thickness Skin Grafts

Tissue damage caused by exposure to subfreezing temperatures and the process of ice recrystallization represents a major limitation in the application of organ cryopreservation for transplantation purposes. A thawing process that does not occur at an optimal rate can trigger excessive ice recrystallization, resulting in cellular and tissue damage, as the formation of large ice crystals increases tissue injury. In the epidermal layer of the skin graft, this phenomenon is reflected in variables such as de-keratinization (DK), epidermal integrity (EI), and ballooning of the epithelial cells (BL). Histological changes in the dermis are indicated by alterations in the dermo- epidermal junction (DEJ), connective tissue fibers breakage (CFB), hair follicle integrity (HF), dermal glands appearance (DGL), and blood vessel structure (BV). In the hypodermis, observed parameters include adipose tissue integrity (AD) and smooth muscle structural changes (SM).[9]

Storage of split-thickness skin grafts is a critical step in wound management, particularly in burn injuries, degloving injuries, and other reconstructive surgical procedures. The most commonly used storage temperatures in clinical practice are 4°C for short-term preservation and ultralow temperatures (−80°C) for long-term preservation using cryopreservation techniques.

The findings of this study show that storage at 4°C in standard media (0.9% NaCl) resulted in no damage (EI) to mild damage in the epidermis and dermis, although the dermo-epidermal junction exhibited severe damage. In contrast, storage at the same temperature using glycerol media preserved more structures without damage (DK and DGL), and no structure exhibited severe damage.

Although the structural integrity remains relatively stable, cellular proliferation decreases substantially, with studies reporting up to a 50% reduction in cell viability by day three and apoptosis increasing by up to 25% by day seven. This indicates that while the physical structure appears preserved, cellular activity decreases overall).[10,11]

This study also shows that storage at −80°C with standard media (0.9% NaCl) resulted in severe damage to the epidermis (DK), although many other structures were preserved or only mildly damaged. Storage using glycerol media yielded better results, with many structures showing no damage (DK, BL, and DEJ).

Literature suggests that storage at−80°C using cryopreservation techniques can preserve tissue for a significantly longer period, with metabolic activity remaining very low. However, the formation of ice crystals during freezing can cause serious microscopic damage, including disorganization of the dermo-epidermal junction, extracellular matrix degradation, and loss of cellular integrity, which may compromise graft function.[11,12] The use of cryoprotectants such as dimethyl sulfoxide (DMSO) may reduce these effects, but the risk of damage remains and may have clinical implications.[13]

Histological studies comparing fres split-thickness grafts with those stored at 4°C and those frozen at −80°C have found that grafts stored only at 4°C tend to retain epidermal layer structure with minimal changes, whereas cryopreserved grafts show mild inflammatory infiltration, more pronounced edema, and increased tissue fragility macroscopically after thawing.[13,14] Immunohistochemical staining techniques such as Ki67 (proliferation marker) and TUNEL (apoptosis) further support these findings, showing significantly better preserved cell proliferation in grafts stored at 4°C compared to those stored at −80°C without optimal cryoprotectant treatment.[15]

Furthermore, −80°C cryopreservation also affects the microvasculature of the graft. Storage at 4°C generally preserves microvascular structures, enabling faster revascularization after transplantation, whereas frozen storage tends to damage vascular endothelial cells, potentially delaying or impairing graft take. This has important implications for clinical readiness, as grafts stored at 4°C are typically ready for immediate use, while cryopreserved grafts require thawing and specific reconditioning protocols.[16]

Tissue Viability

Tissue viability in split-thickness skin grafts depends on storage conditions, storage medium, duration, and temperature. Viability refers to the ability of cells to survive and maintain metabolic function, which is essential for successful transplantation and wound healing.At 4°C, tissue

viability gradually declines over time. Studies using MTT assays and colony-forming efficiency tests to evaluate keratinocyte viability and proliferation have shown approximately a 50% decrease in viability after 3 days of storage, which may drop below 20% after 7– 10 days.[17] This decline correlates with reduced metabolic activity and increased apoptosis in epidermal cells. Nonetheless, 4°C storage remains the preferred method for short-term preservation because it is simple and maintains adequate cellular integrity for several days prior to transplantation.[18] In contrast, viability at −80°C depends heavily on the cryopreservation protocol used. Storage at this temperature without appropriate cryoprotectants typically results in severe reductions in viability due to ice crystal–induced damage during freezing and thawing.[19] However, with the use of DMSO or glycerol as cryoprotectants and optimized protocols, cellular viability can be preserved for months or even years. Clinical and experimental studies on animal models have shown that cryopreserved grafts can achieve engraftment and functional outcomes comparable to fresh grafts when thawing procedures are carefully performed.[19,20] The findings of this study are consistent with the literature, demonstrating that decreasing storage temperature correlates with significantly increased tissue viability. Glycerol is a commonly used cryoprotectant for allograft skin preservation. It reduces ice crystal formation during freezing and prevents osmotic stress that can damage cell membranes and dermal collagen. Although glycerol causes substantial loss of cellular viability due to its dehydrating effects, skin stored in glycerol largely preserves extracellular matrix structure and dermal components essential for engraftment and healing.[21,22] This study similarly found that glycerol storage resulted in higher tissue viability compared to 0.9% NaCl.

Clinically, maintaining high tissue viability is crucial for successful transplantation, because reduced viability may lead to graft failure, chronic wounds, and infection. Therefore, understanding the impact of different storage temperatures on tissue viability is essential in clinical practice.

Study Limitations This study focused on the effects of temperature on split-thickness grafts using in-vitro methods. The study was not conducted directly in living organisms; therefore, the clinical benefits of these temperature effects cannot be confirmed. Long-term effects on different tissue types may not be detectable within the short storage duration used in this study.

CONCLUSION

Split-thickness skin grafts preserved at –80°C exhibited superior histological structure and significantly higher viability compared with grafts stored at 4°C. These findings indicate that ultra-low–temperature preservation is more effective in maintaining the biological integrity of split-thickness skin grafts. Future research should incorporate in vivo experimental models, such as animal studies, prior to conducting comparative effectiveness assessments in human tissues to better understand physiological responses and translational relevance. Longitudinal evaluations at predetermined storage intervals are recommended to determine the extent to which prolonged preservation impacts tissue viability and structural integrity. Such time-based assessments may provide valuable insights into the durability and optimal shelf-life of preserved grafts.

DECLARATIONS

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Competing and conflicting interest:
There are no conflicts of interest.

Consent for publication:
Not applicable. This work was conducted on archival tissue blocks.

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