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# COMPARISION OF CALORIC INTRAVENOUS SOLUTIONS FOR HYPOTHERMIC PRESERVING OF VIETNAMESE STEM CELLS FROM EXFOLIATED DECIDUOUS TEETH

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## ABSTRACT

Mesenchymal stem cells from human exfoliated deciduous teeth (SHED), providing significant potential as regenerative medicine due to their multipotency, immunomodulatory properties, and non-invasive collection process. Effective preservation strategies are essential to maintain their quality during storage and transportation for clinical applications. This study evaluated the effects of two caloric intravenous solutions, Dextrose 5% (D5) and Dextrose 5% in Lactated Ringer (D5LR), on the hypothermic storage of SHED derived from Vietnamese donors. SHED was cultured and characterized for immunophenotyping, as well as osteogenic and adipogenic differentiation potential, prior to preservation. The cells were stored at 4°C or 25°C in D5 or D5LR for durations of 2, 4, and 6 hours, and post-storage assessments included cell viability, adhesion, and proliferation capacity. Results showed that D5 preserved higher cell viability and adhesion rates, with storage at 25°C achieving a 75% initial survival rate within 4 hours before declining significantly at 6 hours. Storage at 4°C maintains better adhesion, while proliferative capacity remained unaffected under both conditions. In contrast, SHED stored in D5LR exhibited lower viability, with dramatic declines in adhesion and a complete loss of proliferative capacity under both storage temperatures. In conclusion, D5 proved effective for short-term hypothermic storage of SHED, preserving key cellular properties critical for regenerative applications. Conversely, the solution of D5LR significantly compromised cell functionality, underscoring its unsuitability for clinical use. Notably, this study also represents the first reported successful isolation of SHED from Vietnamese dental tissue, further enhancing its significance. Additionally, these findings support D5 as a practical solution for preserving SHED quality in regenerative medicine.

**Keywords:** Caloric intravenous solutions, human exfoliated deciduous teeth, hypothermic preservation, mesenchymal stem cells.

## INTRODUCTION

SHED represent a distinct and promising subset of mesenchymal stem cells (MSCs), characterized by their exceptional regenerative potential and broad differentiation capacity (Miura *et al.*, 2003). SHED exhibited an elevated proliferation rate and a greater capacity for population doublings, suggesting a population of multipotent stem cells that may possess a more immature phenotype compared to previously studied postnatal stromal stem cell populations (Miura *et al.*, 2003). Additionally, these cells exhibit multipotency, capable of differentiating into adipogenic, osteogenic, neurogenic, and chondrogenic lineages within ideal conditions. Unlike traditional MSCs, SHED do not directly differentiate into osteoblasts but instead create osteoinductive environments by recruiting host osteogenic cells. This unique capability enhances their utility in bone regeneration and repair, including applications for craniofacial reconstruction and periodontal regeneration (Martinez Saez *et al.*, 2016).

First identified in exfoliated primary teeth, SHED provide a non-invasive, painless, and ethical source of stem cells, making them particularly attractive for research and therapeutic applications (Arora *et al.*, 2009). SHED is a promising derivation of mesenchymal stem cells, offering extensive applications in regenerative medicine. In dental pulp tissue engineering, SHED have exhibited regenerative capability on functional dental pulp by differentiating into odontoblast-like cells. These cells are capable of depositing a mineralized matrix comparable to natural dentin, enabling the restoration of damaged teeth and supporting root development (Wang *et al.*, 2010). SHED's neural crest origin also makes them suitable for neural tissue engineering. Studies have shown their potential to differentiate into neural-like cells, survive in neural environments, and express neural markers, which opens avenues for treating conditions such as Alzheimer's disease, Parkinson's disease and injuries to the spinal cord (Sugimura-Wakayama *et al.*, 2015). Furthermore, SHED-conditioned media have been effective in promoting axon density and nerve regeneration in peripheral injuries (Nakamura *et al.*, 2009). In hepatogenic applications, SHED can differentiate into hepatocyte-like cells under appropriate conditions, expressing markers such as albumin and displaying functional capabilities like urea production and glycogen storage (Ishkitiev *et al.*, 2012). SHED also play a significant role in modulating immune responses by inducing tolerogenic dendritic cell phenotypes and expanding CD4+ regulatory T cells, which are crucial for managing inflammatory and immune-mediated conditions such as autoimmune diseases and graft-versus-host disease (Silva *et al.*, 2014). Their accessibility and ethical procurement make SHED an invaluable tool for advancing innovative treatments across multiple medical fields.

The effective preservation of SHED is essential for their potential use in both research and clinical applications. Advanced cell preservation technologies are pivotal in ensuring the viability and functionality of cells during storage, enabling their application in scientific studies and therapeutic interventions. Preservation methodologies are typically classified based on storage temperature, including hypothermic preservation (1°C to 35°C) (Robinson *et al.*, 2014) and cryopreservation

(-80°C to -196°C) (Liu *et al.*, 2019). Hypothermic preservation, commonly referred to as "shelf-life" preservation, is primarily employed for short-term preservation of biological materials, typically lasting less than three days (Robinson *et al.*, 2014). This method is particularly valuable for biological specimens requiring temporary storage while maintaining their structural and functional integrity. Under hypothermic conditions, cellular metabolism and oxygen consumption are significantly decreased, inducing a state termed "cell pausing" (Robinson *et al.*, 2014). This metabolic reduction allows for the effective preservation of cells for diagnostic and therapeutic purposes. Consequently, hypothermic storage is widely employed for the short-term preservation of biological materials, including cells, tissues, and blood samples. A critical physiological effect of hypothermia is its ability to slow cell cycle progression, thereby minimizing energy demands and alleviating metabolic stress during storage (Rubinsky, 2003). In comparison to cryopreservation at ultralow temperatures, hypothermic preservation offers distinct advantages. It simplifies the processes of harvesting, storing, and retrieving biological materials, making it an accessible and practical solution for facilities with limited resources. Unlike cryopreservation, hypothermic storage eliminates the need for specialized ultra-cold storage systems, thereby reducing costs and logistical complexities. This feature is especially beneficial in resource-constrained environments, such as rural healthcare centers or combat zones, where infrastructure for ultra-cold storage is unavailable (Yang *et al.*, 2020). Furthermore, hypothermic preservation utilizes non-toxic protective agents, mitigating chemical exposure risks and facilitating compliance with regulatory standards. Despite these advantages, the application of hypothermic storage for SHED remains inadequately explored, particularly concerning optimal conditions to maintain their viability and functionality. Addressing these gaps is critical to unlocking the full potential of SHED in research and clinical settings.

Intravenous (IV) fluids are sterile solutions administered directly into the bloodstream to maintain or restore fluid balance, supply essential nutrients, or deliver medications. These fluids are categorized based on their properties into crystalloids and colloids. Crystalloids, such as saline and Lactated Ringer's solution, contain small molecules that easily pass through cell membranes, making them ideal for rehydration and electrolyte replenishment. Colloids, such as albumin or dextran, contain larger molecules that remain in the vascular system, helping to expand blood volume and maintain oncotic pressure. IV fluids are widely used in medical settings for managing dehydration, electrolyte imbalances, shock, and medication delivery, serving as a cornerstone in critical care and surgical interventions (Ismail & Elbaih, 2020). Among these, caloric intravenous solutions are fluids designed to provide hydration along with a source of calories to meet the energy needs of patients. These solutions typically contain dextrose (a form of glucose) in varying concentrations, which serves as a readily available energy source for the body. They are commonly used in situations where oral intake is limited or not possible, such as during fasting, surgery, or critical illness. By supplying calories alongside fluids, these solutions help prevent catabolism and support metabolic demands (Ismail & Elbaih, 2020). D5 is a caloric

intravenous solution that provides hydration and a modest energy source. It contains 5% dextrose, delivering glucose as a readily available calorie supply to meet basic metabolic needs, particularly in patients who cannot eat or drink adequately. The solution is isotonic upon administration but becomes hypotonic as the dextrose is metabolized, allowing for effective hydration at the cellular level. D5 is commonly used to prevent catabolism in fasting or critically ill patients, to correct mild dehydration, and as a carrier fluid for medications. Its versatility and ability to supply calories make it a valuable option in a variety of clinical settings (Upadhyay *et al.*, 2006). On the other hand, D5LR is primarily a caloric intravenous solution designed to deliver hydration and energy simultaneously. The 5% dextrose component provides glucose, an essential calorie source, to support the body's metabolic energy needs, particularly in patients unable to consume food orally. This energy supply helps prevent catabolism and maintains cellular function during periods of stress or fasting. As a caloric solution, D5LR also includes the added benefit of Lactated Ringer's, which restores electrolyte balance and offers lactate to aid in buffering metabolic acidosis. Its role as a caloric intravenous fluid makes it ideal for managing energy deficits in perioperative care, critical illness, and recovery phases (Ismail & Elbaih, 2020).

In this research, we examined the capability of D5 and D5LR as media for the hypothermic preservation of SHED. Key parameters assessed include the surface phenotype, differentiation capacity, viability, adhesion, and proliferation rates of SHED following storage. By focusing on SHED derived from Vietnamese donors, this study also seeks to bridge the representation gap in research and enhance the utility of SHED in personalized medicine. These findings aim to establish an optimized solution for preservation, particularly in patients who might benefit from stem cell transplantation and also require caloric intravenous solutions, such as tissue damage, systemic inflammation, or critical illness.

## MATERIALS AND METHODS

### Source and culture of SHED

SHED was obtained from a 7-year-old female donor at Phuong Dong General Hospital. The cells were supplied by the Stem Cell and Gene Therapy Applied Research Center (StemRec) and utilized as the main biological resource in this study.

SHED was cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, USA) enriched with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA). Cells were seeded into T175 culture flasks (Eppendorf, Germany) and incubated at 37°C in a humidified environment with 5% $CO_2$. Culture medium was then replaced every 2–3 days to ensure optimal viability and cell growth. Once cells achieved 70–80% confluency, they were harvested using trypsin-EDTA (Gibco, USA) and subcultured at defined densities for further use. SHED between passages 3 and 5 were employed for all experimental procedures.

### Immunophenotyping characterization of cultured SHED

Immunophenotypic characterization was conducted following an established protocol (Dominici *et al.*, 2006). SHED cell pellets were rinsed with PBS, centrifuged, then resuspended in PBS. Cells were then incubated in a 100 µL solution containing

CD45-APC, CD90-FITC, and CD105-APC-A750 antibodies (BioLegend, USA) at room temperature for 30 minutes. Post-incubation, the cells were rinsed with PBS and analyzed using a MACSQuant® VYB flow cytometer (Miltenyi Biotec, USA). Data were processed using MACSQuantify™ software (Miltenyi Biotec, USA).

**Differentiation assay of cultured SHED**

For osteogenic differentiation, SHED were cultured in OsteoDiff medium (StemMACS, USA) for 14 days with the medium changed every 3 days. Following differentiation, cells were washed twice with PBS, then fixation was performed with 4% formaldehyde for 15 minutes, and stained using Alizarin Red (Sigma-Aldrich, USA) for visualization of calcium deposition.

For adipogenic differentiation, SHED were incubated in AdipoDiff medium (StemMACS, USA) for 14 days, with the medium replaced every 3 days. At the end of differentiation, PBS was used twice to wash the cells, then fixation was performed with 4% formaldehyde for 15 minutes, and stained with Oil Red O (Sigma-Aldrich, USA) for 60 minutes to determine lipid vacuoles.

**Cell viability assay**

Passage 3 SHED were utilized to evaluate cell viability. PBS was used to wash the cells, then the cells were detached using 0.25% rypsin-EDTA, and resuspended at a density of $10^6$ cells/mL. For hypothermic preservation, SHED were stored in either D5 (Fresenius Kabi, Vietnam) or D5LR (Fresenius Kabi, Vietnam). Viability was assessed at 2, 4, and 6 hours of storage at 4°C and 25°C. Trypan blue (Sigma-Aldrich, USA) staining was used to evaluate cell viability as per the manufacturer's protocol. Fresh cells served as controls.

**Cell adhesion assay**

Cell adhesion was examined following a previously reported method (Chen *et al.*, 2013). After 6 hours of storage, 96-well plates were employed to seed 100 µL of culture medium containing $2 \times 10^4$ cells and incubated for 24 hours at 37°C in 5% $CO_2$. The wells were washed twice with PBS, then the cells were fixed for 10 minutes using formaldehyde, and subsequently stained for 10 minutes using 0.5% crystal violet. The extent of adhesion was quantified at 550 nm using a Multiskan™ FC Microplate Photometer (Thermo Scientific, USA).

**Population doubling time**

Following 6 hours of storage, 24-well plates were used to seed SHED (Corning, USA) at a density of $10^4$ cells/well. Subsequently, the cells were cultured to approximately 80% confluency across three consecutive passages. The following formula was used to determine the population doubling time (PDT) (Rizal *et al.*, 2021):

$$\mathrm{PDT} = \mathrm{t} \times \frac{\log(2)}{\log(\mathrm{N2}) - \log(\mathrm{N1})}$$

In the formula, t denotes the total culture duration, N1 denotes the initial cell number, and N2 signifies the final cell number.

**Statistical analysis**

All data were expressed as mean ± standard deviation (SD). One-way and two-way ANOVA were employed for statistical comparisons, followed by Dunnett's multiple comparison tests. A *p*-value of less than 0.05 was considered statistically significant.

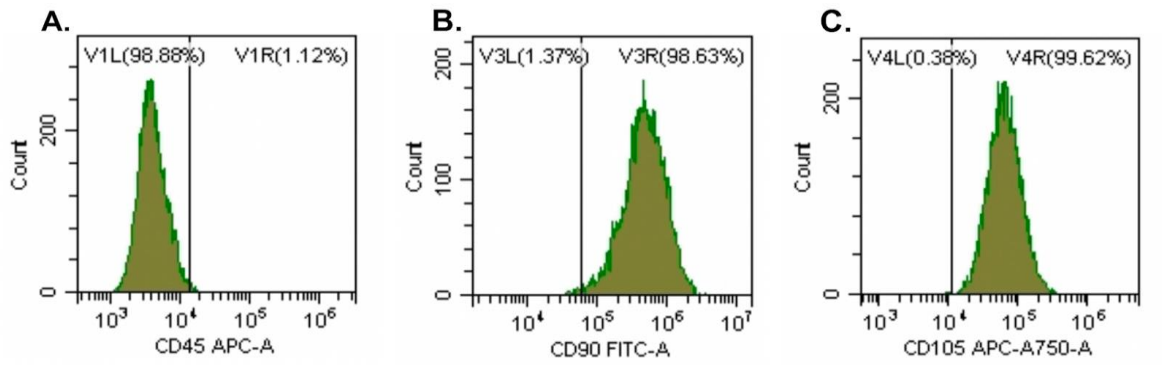


**Figure 1.** Phenotypic characterization of SHED by flow cytometry analysis. Histograms show the lack of expression of CD45 (A), high expression of CD90 (B) and CD105 (C).

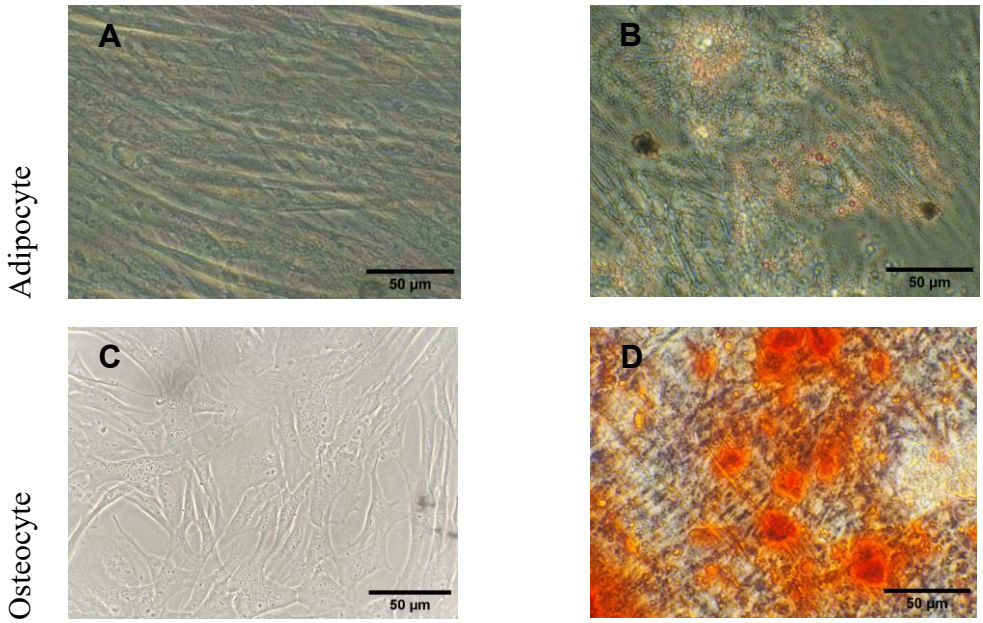


**Figure 2.** Adipogenic and osteogenic differentiation of SHED. Pre- (A) and mature adipocytes (B) were stained with Oil Red O; Pre- (C) and mature differentiated osteocytes (D) were examined by Alizarin red staining, visualized under a light microscope (40 x).

## RESULTS

### Characterization of cultured SHED

The characterization of SHED was comprehensively conducted through immunophenotyping and differentiation assays, confirming their MSC's identity and differentiation potential. Using flow cytometry analysis, it was discovered that SHED lack the hematopoietic marker CD45 (Figure 1A), a defining characteristic that distinguishes them from hematopoietic cell populations. Instead, SHED exhibited strong expression of classical MSC markers, with CD90 detected in 98.63% of the population (Figure 1B) and CD105 expressed in 99.62% (Figure 1C). This high-level expression of

MSC-specific markers reflects a highly pure MSC phenotype, consistent with established MSC characterization criteria. The absence of CD45 further underscores the non-hematopoietic origin of SHED, affirming their lineage specificity and suitability for applications in regenerative medicine.

The differentiation assays further validated the functional capabilities of SHED, demonstrating their ability to undergo lineage-specific differentiation into adipocytes and osteocytes under appropriate induction conditions (Figures 2A-D). Oil Red O staining of SHED-derived adipocytes confirmed successful adipogenic differentiation by highlighting the presence of abundant lipid vacuoles within the cells. Similarly, osteogenic differentiation was evidenced by robust calcium deposition, visualized through Alizarin Red staining, indicating the formation of a mineralized matrix during the differentiation process.

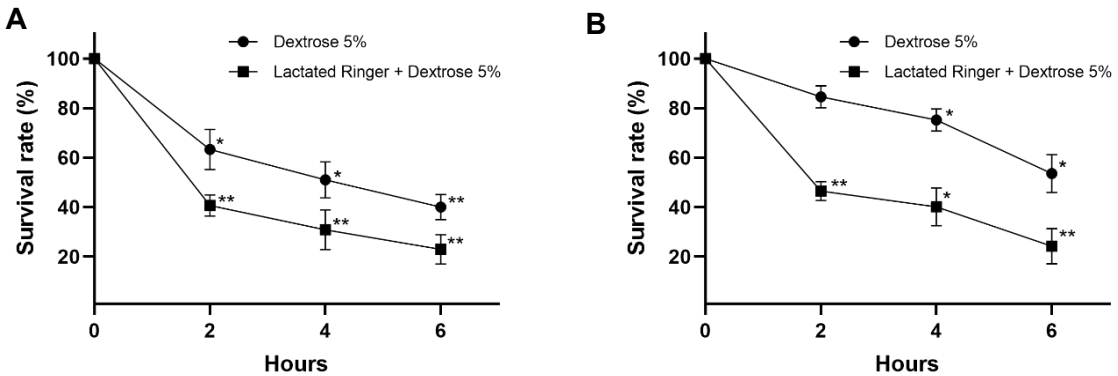


**Figure 3.** Viability assessment of SHED under tested preservation conditions for up to 6 hours. Viability rate for SHED at 4°C (A) and room temperature (B) at 2, 4, and 6 hours. n = 3 per condition, mean ± SD, *p < 0.05, **p < 0.01 compared with the value at time 0 by two-way ANOVA followed by Dunnett multiple comparisons.

## Cell viability of post-storage SHED

Cell viability is a fundamental factor in cell transplantation, as it directly impacts the success and efficacy of therapeutic outcomes. The viability of SHED was assessed under various storage conditions, revealing significant differences influenced by temperature and solution composition. As illustrated in Figure 3A, SHED stored in D5 solution at room temperature initially maintained a viability of 75% during the first 4 hours. However, this value declined sharply to 53.53 ± 7.64% by the final hours of observation (Figure 3B). In contrast, SHED stored at 4°C exhibited a slower reduction in viability, starting at 63.27 ± 8.05% at 2 hours and decreasing to 40.02 ± 5.11% by 6 hours. These results underscore the protective effect of hypothermic storage in mitigating viability loss over time.

Among the storage solutions tested, D5LR demonstrated the lowest survival rates under both temperature conditions. At 4°C, SHED viability in D5LR dropped significantly to 22.92 ± 5.9% after 6 hours, while room temperature storage resulted in a similar decline, with viability reaching 24.21 ± 7.13% over the same period (Figures 3A-B). These findings indicate that D5LR is less effective at maintaining SHED viability compared to D5 under identical conditions.

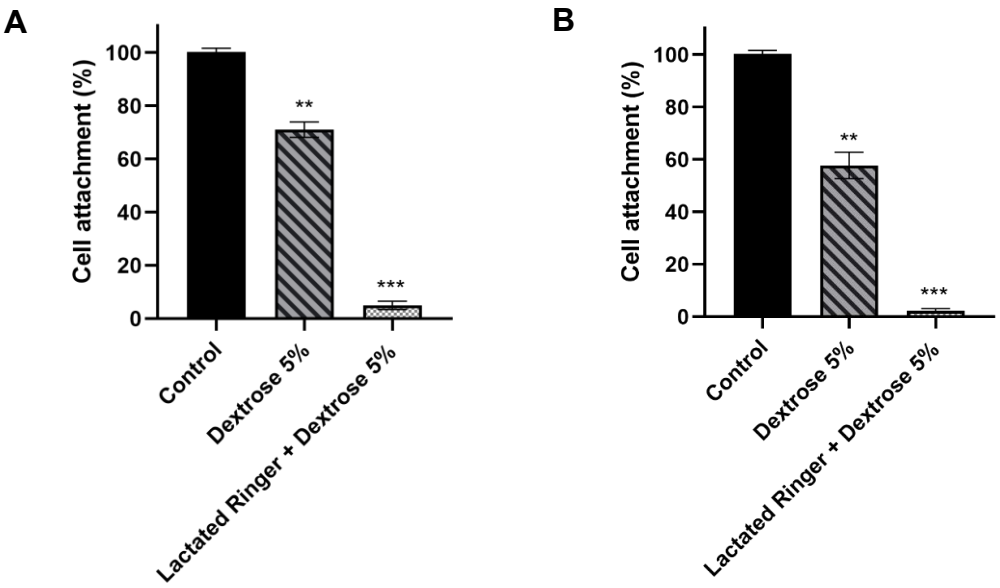


**Figure 4.** Cell adhesion assay for SHED suspended in the different intravenous solutions for 6 hours at 4°C (A) and at room temperature (B). n = 3 per condition, mean ± SD, **p < 0.01, ***p < 0.001 compared to control sample by one-way ANOVA followed by Dunnett multiple comparisons.

## Cell adhesion of post-storage SHED

This experiment was designed to assess the effects of hypothermic preservation on the adhesion capacity of SHED, a crucial parameter for evaluating cell quality in transplantation. Adhesion capacity directly influences cell survival, functionality, tissue integration, and overall therapeutic potential (Ahmad Khalili & Ahmad, 2015; Chen *et al.*, 2013). The results, as presented in Figure 4, reveal that hypothermic preservation significantly reduced the adhesion ability of SHED compared to the control group, with notable variations depending on the storage conditions.

SHED preserved in D5 solution at 4°C demonstrated the highest adhesion rate, retaining 71.03 ± 2.87%, which underscores the protective effect of low-temperature storage in maintaining adhesion-related properties. However, when stored at 25°C in D5, the adhesion rate decreased to 57.70 ± 5.04%, indicating that room temperature storage is less effective in preserving SHED adhesion. In stark contrast, SHED stored in D5LR exhibited a dramatic loss of adhesion capacity under both conditions. At 4°C, adhesion rates plummeted to just 5 ± 1.54%, while at 25°C, the rate further declined to 2.28 ± 0.76%.

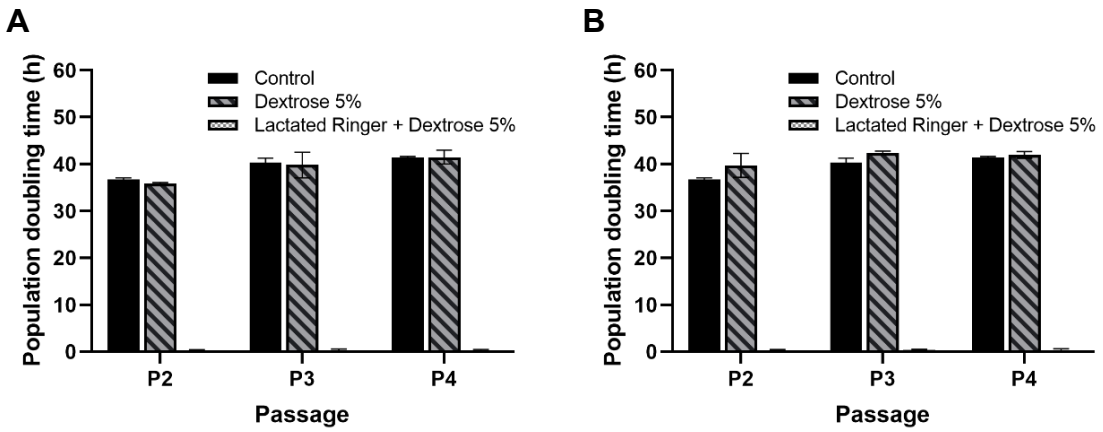


**Figure 5.** Post storage growth potential for SHED suspended in different solutions for 6 hours at 4°C (A) and room temperature (B) for three successive passages. n = 3 per condition, mean ± SD, *p < 0.05 compared to the control sample by two-way ANOVA followed by Dunnett multiple comparisons.

## Population doubling time of post-storage SHED

This experiment investigated the impact of post-storage conditions on the proliferative capacity of SHED, a key determinant of their therapeutic viability. Proliferative capacity was assessed using population doubling time analysis, as shown in Figure 5. The results revealed that SHED stored in D5 solution maintained their proliferative ability across three passages, with no significant changes in PDT observed under either 4°C or room temperature conditions. This consistency highlights the effectiveness of D5 in preserving SHED's proliferative potential during hypothermic storage. In contrast, SHED stored in D5LR exhibited a complete cessation of proliferation, with no observable cell growth in any subsequent passages.

## DISCUSSION

In this study, we investigated the effects of storage solutions on the survival, proliferation, and attachment characteristics of SHED. Our results revealed that SHED stored in D5 at 25°C for 4 hours maintained a survival rate exceeding 70%, a finding that complies with FDA regulations for cell transplantation. Furthermore, SHED preserved in D5 exhibited no significant decrease in proliferation capacity, although their attachment capabilities were notably diminished. In contrast, SHED stored in D5LR failed to sustain survival, proliferation, or attachment, highlighting the importance of optimizing storage conditions for effective transplantation.

SHED previously reported that these cells express the cell surface molecules STRO-1 and CD146 (MUC18) – two early MSC markers. Phenotypic analyses revealed the presence of various MSC surface markers, notably CD146 and STRO-1, as well as CD10, CD44, CD90, CD166, CD29, CD71, CD73, CD105, CD117, and SSEA-4. However, SHED lacks the expression of CD197, CD133, CD49e, CD45, CD31, CD7, CD34, CD106, CD184 and HLA-DR (Yamaza *et al.*, 2010). These findings indicate that SHED are not derived from hematopoietic lineages and are pure MSCs if the cells cannot express CD45 and CD34

(signature markers of hematopoietic stem cells), CD7, a T-cell marker and HLA-DR, an immune cell marker. Furthermore, SHED appear to exhibit a less differentiated state compared to DPSC, as indicated by their positive expression of CD117 (receptor for stem cell factor I, a characteristic of pluripotent cells) and endothelial differentiation markers such as CD106 and CD31 (Suchánek *et al.*, 2010). Finally, the positive expression of CD71 (appeared in proliferating cells) and the endothelial cell marker endoglin, or CD105 supports the idea that SHED exhibits superior proliferative potential in comparison with the DPSC lines (Miura *et al.*, 2003). Our results suggested that culture conditions did not significantly impact the expression of the surface markers of CD105, CD73 and CD90.

SHED demonstrate significant potential to differentiate into multiple cell types, particularly in the osteogenic, chondrogenic, and adipogenic lineages, making them a promising resource for regenerative medicine. In the osteogenic lineage, SHED have been observed to differentiate into osteoblasts, expressing key bone markers such as bone sialoprotein, alkaline phosphatase, osteocalcin, matrix extracellular phosphoglycoprotein, core-binding factor-α-1 and especially the transcriptional factor critical for the formation of bone and osteoblast differentiation (Miura *et al.*, 2003). Studies utilizing hydroxyapatite and tricalcium phosphate scaffolds demonstrated SHED's capacity to contribute actively to bone regeneration, repairing critical-size defects and maintaining bone continuity for extended periods (Seo *et al.*, 2008). Furthermore, SHED have been shown to promote differentiation into osteogenic cells in recipient cells while actively contributing to new bone formation, highlighting their therapeutic potential for craniofacial and skeletal defect repair (Huang *et al.*, 2009). SHED also exhibit chondrogenic differentiation potential. *In vitro*, SHED cultured in chondrogenic induction media containing components such as transforming growth factor-β and basic fibroblast growth factor demonstrated upregulated expression of chondrogenic markers like SOX9, collagen II, and collagen X (Koyama *et al.*, 2009). When transplanted with β-TCP scaffolds *in vivo*, SHED generated cartilage-like tissues, confirming their ability to differentiate into chondrocytes and suggesting potential applications in cartilage tissue engineering (Annibali *et al.*, 2014). Adipogenic differentiation of SHED has been evidenced through the formation of lipid-laden fat cells and the upregulation of adipocyte-specific markers such as peroxisome proliferator-activated receptor-γ2 and lipoprotein lipase (Koyama *et al.*, 2009; Miura *et al.*, 2003). However, some studies have indicated that SHED may have reduced adipogenic potential compared to their robust osteogenic capabilities, as shown by decreased lipid-specific staining and expression of adipocyte-specific molecules (Yamaza *et al.*, 2010). Our results suggested that culture conditions did not significantly impact the functional characteristics of MSCs in differentiation into adipocytes and osteocytes.

Hypothermic preservation, which utilizes temperatures below the physiological norm of 37°C, is a commonly employed technique to reduce the metabolic rate of cells and other biological materials, allowing for their short-term storage and transportation (Kamijima *et al.*, 2013). While hypothermia in humans, a drop in body temperature below 35°C, is considered a life-threatening

condition, isolated human cells exhibit a remarkable ability to tolerate and survive in colder conditions. This tolerance arises from the fundamental biochemical principle that cellular reactions follow an Arrhenius relationship with temperature, meaning reaction rates decrease exponentially as temperatures drop (Agarwal *et al.*, 2005). These variations indicate that the oxygen demands during storage are highly cell-type dependent, reflecting the diverse metabolic needs of various biological systems. Furthermore, as intracellular oxygen stores are inherently limited, a reduction in oxygen uptake rate is accompanied by a general decrease in metabolic activity, subsequently reducing the demand for other nutrients. This necessitates the use of specialized storage media tailored to the unique conditions of hypothermic preservation, distinct from those used under standard culture conditions (Dalton *et al.*, 1999). Despite its benefits, hypothermic preservation does not entirely halt biochemical reactions at temperatures above 0°C, requiring meticulous management to prevent damage while maintaining viability (Taylor, 2006). Research demonstrates that extremely low temperatures, such as 1°C and 5°C, can induce significant cellular stress, leading to increased rates of apoptosis and necrosis compared to higher preservation temperatures like 16°C and 22°C (Wang *et al.*, 2017). This underscores a critical trade-off in hypothermic preservation: while lower temperatures reduce metabolic activity and the need for nutrient supplementation, they simultaneously elevate the risk of cell death. The composition of the preservation medium plays a pivotal role in mitigating these effects. For instance, glucose supplementation at 4°C has been found to reduce the recovery of functional immature cardiomyocytes and increase the release of lactate dehydrogenase, signaling cellular damage (Orita *et al.*, 1994). However, glucose enhanced ATP and ADP concentrations, improving cell survival during short-term hypothermic storage. Further supplementation with fructose-1,6-bisphosphate boosted these energy levels, although its efficacy diminished over extended storage periods, with only minor effects observed on ATP levels after 48 hours (Wheeler *et al.*, 2005). One of the key challenges in hypothermic preservation is managing the imbalance between ATP supply and demand, which is a primary driver of cell death at reduced temperatures. Mitochondrial ATP production slows dramatically under hypothermic conditions, yet cells retain a residual energy demand, leading to accelerated ATP depletion (Maathuis *et al.*, 2007). Within hours, ATP levels can decrease by as much as 95%, impairing essential cellular functions such as $Na^+/K^+$ ATPase activity (Maathuis *et al.*, 2007). This impairment disrupts ionic homeostasis, resulting in membrane depolarization, cytoskeletal instability, and dysregulated ion fluxes of $Na^+$, $K^+$, $Cl^-$, $Ca^{2+}$, and $Fe^{2+}$ (Guibert *et al.*, 2011). These ionic imbalances are often accompanied by osmotic disturbances, excessive water influx, and subsequent cellular swelling, which further reduces cell viability. Strategies to counteract these effects include the addition of ATP precursors like adenosine and phosphate ions to preservation media, which have shown promise in mitigating ATP depletion and improving cellular outcomes during hypothermic storage (Lockett *et al.*, 1995). Although hypothermic preservation effectively slows cell cycle progression and prevents ice formation, it introduces risks such as hypothermia-induced injuries and extracellular matrix (ECM) loss, which can cause irreversible cellular damage (Köllmer

*et al.*, 2012). ATP depletion and reduced enzymatic activity under hypothermic conditions disrupt cellular metabolism and redox balance, triggering a cascade of adverse events (Cosby *et al.*, 2008). While hypothermia slows biochemical reactions and reduces metabolic demands, these remaining reactions must be carefully managed to avoid damage and maintain cell viability. Thus, hypothermic preservation, typically conducted at temperatures between 1°C and 35°C, remains an indispensable method for short-term storage of biological samples. Continued research into optimizing storage media, incorporating metabolic supplements, and mitigating hypothermia-induced injuries holds great potential to enhance the efficacy and reliability of this technique (Cosby *et al.*, 2008; Rubinsky, 2003). Our study observed that D5 demonstrated superior preservation of SHED at both temperatures. D5 has an osmolarity of 278 mOsm/L, which approximates physiological osmolarity (286 mOsm/L), which greatly maintains the cell survival rates within 4 hours. However, its glucose content depletes rapidly *in vitro*, potentially leading to decreased SHED viability after 4 hours at 25°C (Nuschke *et al.*, 2016). In contrast, the D5LR resulted in a marked decrease in cell survival due to the higher osmolarity of this mixture (525 mOsm/L) compared to physiological levels, leading to osmotic stress and damage (Zhang *et al.*, 2022).

Maintaining the proliferative capacity of MSCs is essential for the efficacy of transplantation, as it guarantees an adequate supply of cells capable of contributing to tissue repair, regeneration, and immunomodulation. The post-transplantation expansion of MSCs enables their differentiation into numerous cell types, consisting of adipocytes, osteoblasts, and chondrocytes, which are vital for the regeneration of damaged tissues. Moreover, the preservation of MSC's proliferative potential is imperative for their role in modulating immune responses, optimizing therapeutic outcomes, and sustaining long-term tissue homeostasis. A compromise in proliferation can significantly undermine the therapeutic benefits of MSC transplantation and lead to diminished clinical outcomes (Petrenko *et al.*, 2019). Additionally, hypothermic conditions have been shown to inhibit cell proliferation across various cell types, including embryonic and somatic cells, as well as cancer cells (Carmeliet *et al.*, 1998). DNA replication, as measured by bromodeoxyuridine incorporation, decreases particularly in cells from wild-type models exposed to hypoxia. However, this reduction is not observed in cells deficient in HIF-1α, indicating a critical dependence on this factor (Carmeliet *et al.*, 1998). Furthermore, overexpression of HIF-1α alone is sufficient to induce cell cycle arrest (Hubbi *et al.*, 2013). The mechanisms by which HIF-1α mediates this arrest involve both transcriptional pathways, notably through the CDK inhibitors p21 and p27, and non-transcriptional mechanisms, including direct effects on the pre-replicative complex. However, in our investigation, SHED stored in D5 demonstrated proliferation rates comparable to those of fresh cells. In contrast, storage in D5LR solution resulted in irreversible damage due to the high osmolarity of this mixture, eliminating the cells' proliferative capacity (Liang *et al.*, 2012). These findings corroborate earlier studies, which reported that the proliferation of umbilical cord MSCs (UC-MSCs) was unaffected by storage in 5% dextrose, 0.9% saline, or Plasma-Lyte A solutions (Chen *et al.*, 2013).

Cell adhesion is a fundamental process in our biological system, spanning from entire organisms to individual cells. It plays a pivotal role in cell communication, regulation, organogenesis, and tissue maintenance (Deng *et al.*, 2017). As a dynamic and intricate mechanism, cell adhesion involves the adsorption of proteins onto surfaces and the expression of specific peptide sequences. *In vivo*, cells are embedded within ECM, a three-dimensional (3D) network comprising proteoglycans, glycosaminoglycans, adhesion proteins, and fibrin. The ECM provides diverse biochemical and mechanical signals that influence various cell behaviors (Agmon & Christman, 2016). Through integrins, cells adhere to specific surfaces, while those unable to adhere often undergo apoptosis. The ECM contains proteins that are recognized by integrins and other cellular receptors, including arginine-glycine-aspartic acid ligands, fibrinogen, vitamin C protein, collagen, and fibronectin. These ligands orchestrate a wide range of cellular physiological processes, such as adhesion, migration, proliferation, secretion, gene expression, and apoptosis, all of which are modulated by ECM signaling (Seo *et al.*, 2011). Additionally, categorized as transmembrane proteins, integrins are essential to cell adhesion by mediating attachment to the extracellular matrix or other cells through specific adhesion molecules (Saif *et al.*, 2003). This essential process supports cellular function and tissue integrity. The linkage between integrins and the cytoskeleton, particularly the actin filaments, is achieved through focal adhesion complexes, a highly organized network of proteins (Horwitz, 1997). This structural framework not only supports the nucleus but also maintains the cell's shape and plays a pivotal role in mechanotransduction by transferring mechanical forces to the cytoskeleton (Honarmandi *et al.*, 2011). Focal adhesion complexes, formed upon integrin engagement, transmit both adhesive and traction forces, crucial for cell migration, proliferation, and differentiation (Beningo *et al.*, 2001). These processes are essential for tissue organization, maintenance, and repair (Ahmad Khalili & Ahmad, 2015). In *in vitro* studies, cell adhesion is characterized by three distinct phases: initial attachment to the substrate, subsequent spreading and flattening, and the final organization of the actin cytoskeleton into focal adhesions (LeBaron & Athanasiou, 2000). Cell adhesion and the contact between the cell and the substrate are positively correlated, with the determined factor being the duration of the contact (Ahmad Khalili & Ahmad, 2015). Our investigation revealed a notable decrease in cell attachment after 6 hours of storage in D5, while SHED in D5LR showed a complete loss of adhesion capacity. Furthermore, higher storage temperatures during hypothermic preservation exacerbated the reduction in cell attachment. Previous studies have demonstrated that MSCs stored at low temperatures (2 to 8°C) in solutions such as 0.9% sodium chloride or Plasma-Lyte A experience over a 25% decrease in attachment after 6 hours, although this is still higher than attachment rates observed for MSCs stored at room temperature for the same period (Chen *et al.*, 2013). Although the exact mechanisms remain to be fully elucidated, these observations are likely due to reduced metabolic activity and slower ATP depletion at lower temperatures, resulting in higher residual ATP levels, improved metabolic recovery, and enhanced activation of integrins and adhesion

molecules following storage (Ahmad Khalili & Ahmad, 2015).

## CONCLUSION

Our findings demonstrated that SHED cells stored in D5 at 25°C for 4 hours comply with FDA standards for cell transplantation, while the solution of D5LR significantly compromised the post-storage functional properties of SHED. Remarkably, this study marks the first successful isolation of SHED from Vietnamese dental tissue, providing a unique contribution to the field. Furthermore, these results have crucial implications for the clinical application of SHED, as the ability to maintain cell quality during transportation and storage is a critical factor for successful cell-based regenerative therapies. The superior preservation of SHED in D5 at 25°C for 4 hours highlights the importance of optimizing storage conditions to ensure the delivery of high-quality stem cells for therapeutic use. For future research, the optimization of storage solutions and protocols to enhance the viability and functionality of SHED cells during hypothermic storage should be investigated. Additionally, the mechanisms underlying this preservation effects need to be more clearly elucidated to improve our understanding of how different storage solutions affect cell function.

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## AUTHOR CONTRIBUTIONS

TTNN: Investigation. TBCH: Investigation, Data curation, Writing-original draft. VTD: Conceptualization, Investigation, Review & Editing, Supervision. TKHL: Investigation. DSN: Investigation. HHC: Review & Editing, Funding acquisition. TSN: Review & Editing, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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