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HYPOTHERMIC STORAGE OF VIETNAMESE STEM CELLS FROM EXFOLIATED DECIDUOUS TEETH USING CRYSTALLOID ELECTROLYTE SOLUTIONS

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ABSTRACT

Preserving the quality of mesenchymal stem cells (MSCs) during storage is crucial for their effective use in clinical applications, particularly in regenerative medicine and tissue engineering. This study investigated the effects of crystalloid electrolyte solutions on the hypothermic storage of Stem cells derived from Human Exfoliated Deciduous teeth (SHED) of Vietnamese, focusing on maintaining cell viability, proliferation, and adhesion capacities. Before storage, the cells were cultured and characterized for immunophenotypic markers, as well as osteogenic and adipogenic differentiation potentials. They were then stored in Lactated Ringer (LR) and Dextrose 5% in Lactated Ringer's (D5LR) at 4 or 25°C for 2, 4, and 6 hours. Post-storage assessments revealed that LR at 4°C was the more effective solution, preserving cell viability and proliferation ability after 6 hours of storage. However, a significant reduction in cell adhesion was observed under these conditions. Conversely, cells stored in Dextrose 5% plus Lactate Ringer completely lost their capacity to proliferate and adhere to plastic surfaces, indicating its unsuitability for preserving cells. This study marks the first successful isolation of SHED from Vietnamese teeth, while also highlighting the potential of Lactate Ringer at 4°C as an optimal short-term storage solution, ensuring the functional integrity of SHED for clinical transplantation. This approach offers promising applications in the field of regenerative medicine, supporting advancements in therapeutic strategies for tissue repair and engineering.

Keywords: Mesenchymal stem cells, human exfoliated deciduous teeth, hypothermic preservation, crystalloid electrolyte solution, Lactate Ringer, Dextrose.

INTRODUCTION

Mesenchymal stem cells (MSCs) are highly versatile plastic-adherent cells with exceptional self-renewal abilities and the capacity to differentiate into a variety of lineages, such as osteogenic, chondrogenic, adipogenic, and others (Jiang *et al.*, 2007). These cells are characterized by their low immunogenicity and robust immunomodulatory properties, allowing them to interact with the immune system in ways that promote healing while minimizing adverse reactions (Aggarwal & Pittenger, 2005). MSCs can be derived from a wide range of tissues, including bone marrow, adipose tissue, dermal tissue, intervertebral discs, amniotic fluid, dental tissues, human placenta, and cord blood. Their presence across various tissue types highlights their biological significance in maintaining tissue homeostasis and responding to injury or inflammation (Li *et al.*, 2019). These properties make MSCs invaluable in regenerative medicine, where they offer solutions to complex medical challenges.

MSCs have demonstrated remarkable success due to their unique combination of properties, including their ability to "home" to sites of tissue damage, differentiate into multiple cell types, and exert potent immunomodulatory effects (Ullah *et al.*, 2019). They have been used extensively in cartilage and bone repair, where their ability to regenerate skeletal tissues has been pivotal in restoring structural and functional integrity (Granero-Molto *et al.*, 2008). In the context of wound healing, MSCs promote tissue repair by enhancing angiogenesis, modulating inflammation, and accelerating epithelialization. Their contributions to neuronal regeneration are particularly noteworthy, offering new therapeutic possibilities for treating spinal cord injuries, neurodegenerative diseases, and ischemic stroke by supporting the repair and regrowth of damaged neural tissues (Andrzejewska *et al.*, 2021; Shariati *et al.*, 2020). Additionally, their immunomodulatory properties have been successfully utilized in treating immune-related conditions. MSCs have been used in managing graft-versus-host disease, a severe complication of bone marrow transplantation, where their immunosuppressive effects help to mitigate host immune responses and improve patient outcomes. Their ability to fine-tune the immune response further opens doors to treating autoimmune diseases and chronic inflammatory conditions (Kartiko *et al.*, 2017; Li *et al.*, 2024). As research continues to evolve, MSCs are expected to play an increasingly vital role in developing innovative therapies that address unmet medical needs across various domains, including orthopedics, dermatology, neurology, cardiology, and immunology.

A specific subset of MSCs, referred to as SHED, has emerged as a promising candidate in the field of stem cell therapy. SHED, which are derived from exfoliated deciduous teeth, exhibit a broader differentiation potential compared to other stem cell populations, positioning them as valuable resources for regenerative medicine (Arora *et al.*, 2009). The processes of collecting, isolating, and storing SHED are relatively simple and non-invasive, further enhancing their practicality for clinical use. SHED are particularly advantageous due to their wide differentiation capabilities, ease of accessibility, and the non-painful nature of their collection process for both the child and parent. Additionally, the cost associated with SHED storage is significantly lower, less than one-third, compared to cord blood

storage, and SHED could also be beneficial for close relatives of the donor (Arora *et al.*, 2009), with easy accessibility, uncomplicated procedure and pain-free for both child and parent, along with better ethical acceptability compared to other sources that may require more invasive procedures . Importantly, SHED provide a matched donor source for autologous transplants, thereby eliminating the risk of immune rejection. Notably, SHED have indicated a significant capacity to induce bone formation *in vivo* through recipient cell-mediated mechanisms. Rather than differentiating directly into osteoblasts, SHED create an osteoinductive environment that attracts host osteogenic cells. Moreover, SHED have shown the ability to modulate monocyte-derived dendritic cells, inducing an immune-regulatory phenotype through the regulation of their maturation and differentiation, inhibiting lymphocyte stimulation, and expanding CD4+ T cells, highlighting their potential for immune modulation in clinical settings (Silva *et al.*, 2014). These results imply that deciduous teeth may not only guide the eruption of permanent teeth but also play a role in inducing bone formation during this process. Furthermore, SHED express markers associated with neuronal and glial cells, reflecting their neural crest cell origin, which is critical in embryonic development and give rise to numerous cell types, including neural cells, smooth muscle, and craniofacial structures (Chai *et al.*, 2000; LaBonne & Bronner-Fraser, 1999). Although the full biological significance of SHED is yet to be determined, their non-invasive isolation and retention of multipotency after *in vitro* expansion offer significant advantages, making them a key focus for commercial stem cell banks seeking autologous sources for various therapeutic purposes (Bjornson *et al.*, 1999). The escalating interest in SHED has led to advancements in methods designed to maintain high cell quality throughout the process of harvesting and clinical application.

The stability of cell-based medicinal products is fundamental in ensuring the safety and efficacy of functionality in clinical applications. Hypothermic preservation is a short-term preservation method that operates at temperatures ranging from 1 to 35°C. Unlike cryopreservation, which involves ultra-low temperatures, hypothermic preservation maintains cells in an unfrozen state, significantly reducing their metabolic rate and oxygen demand (Freitas-Ribeiro *et al.*, 2019; Karakoyun *et al.*, 2019; Kemp, 2006). This method is widely used for its simplicity, cost-effectiveness, and reliance on non-toxic protectants, making it suitable for short-term storage and transport of cells, tissues, or therapeutic products in settings where ultra-cold storage facilities are unavailable (Brinkkoetter *et al.*, 2008; Ma *et al.*, 2021; Yang *et al.*, 2020). Applications in cell therapy highlight its relevance, particularly in transporting living cells to clinical sites for immediate use, such as a large diversity of cells and tissues, including human pluripotent stem cell-derived cardiomyocytes (Correia *et al.*, 2016), bone marrow mesenchymal stem cells (Ginis *et al.*, 2012), endothelial cells (Post *et al.*, 2013), renal cells (Mathew *et al.*, 2002; Salahudeen *et al.*, 2001), and hepatocytes (Duret *et al.*, 2015; Gramignoli *et al.*, 2014). For regenerative medicine, hypothermic preservation ensures that constructs retain their cellular viability and functionality during transport, facilitating seamless integration into therapeutic workflows. Emerging advancements focus on

biomaterials that regulate ATP levels, stabilize membranes, balance antioxidant defenses, and mimic the extracellular matrix, all of which aim to enhance cell survival during storage and transport (Belzer & Southard, 1988; Heddle *et al.*, 2016; Rubinsky, 2003). This preservation strategy, while effective for short durations, continues to evolve with innovative materials and methodologies, ensuring its vital role in supporting the growing demands of cell-based medicine.

Crystalloid electrolyte solutions are intravenous fluids containing water and electrolytes that closely mimic the body's natural plasma composition. They are widely used in medical settings to restore fluid balance, maintain blood volume, and support tissue oxygenation and nutrient delivery. These solutions are essential in managing dehydration, blood loss, and other acute medical conditions. (Orbegozo Cortés *et al.*, 2014). LR is a balanced isotonic crystalloid fluid widely used in medical practice for fluid replacement therapy. Its composition includes essential electrolytes such as sodium, chloride, potassium, calcium, and lactate in the form of sodium lactate, resulting in an osmolarity of approximately 273 mOsm/L and a pH of about 6.5. This balanced formulation mimics the body's plasma composition, effectively restoring fluid and electrolyte balance while providing a buffering effect through lactate metabolism into bicarbonate (Trujillo-Zea *et al.*, 2015). On the other hand, D5LR is a type of crystalloid electrolyte solution designed to provide both hydration and energy. It combines the benefits of LR, which contains essential electrolytes like sodium, potassium, calcium, and lactate for restoring fluid and electrolyte balance, with 5% dextrose to supply an energy source. The addition of dextrose helps prevent catabolism in fasting or critically ill patients, while lactate acts as a buffer to counteract metabolic acidosis. As a crystalloid solution, D5LR is isotonic and widely used in managing dehydration, electrolyte imbalances, and metabolic needs in various clinical settings (Kaviti *et al.*, 2024).

Currently, there is a lack of research focused on optimizing or validating hypothermic storage conditions for SHED derived from Vietnamese populations. The absence of well-established guidelines for the preservation of SHED under low-temperature conditions could pose significant challenges for a wide range of applications, including regenerative medicine, drug testing, and diagnostic procedures. In this study, LR and D5LR were evaluated for the hypothermic preservation of SHED. The surface phenotype and differentiation capacity of cultured SHED, along with post-storage cell viability, adhesion capacity, and proliferation rate, were also examined.

MATERIALS AND METHODS

Cell source and culture

Stem cells from human exfoliated deciduous teeth were obtained from a 7-year-old female donor at Phuong Dong General Hospital. The cells were provided by the Stem Cell and Gene Therapy Applied Research Center (StemRec, Vietnam), which served as the primary source for this study. SHED are then cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% FBS (Sigma-Aldrich, USA). To initiate culturing, SHED are seeded in T175 flasks (Eppendorf, Germany) inside an incubator with 37°C and

5% CO_2 supplement. Then, the medium is changed every 2 to 3 days to support cell growth and viability. When SHED reach 70 to 80% confluency, they are detached using trypsin-EDTA (Gibco, USA) and reseeded at desired densities for further expansion. SHED at P3 to P5 were used for all the experiments.

Immunophenotyping characterization of cultured SHED

The cell surface markers were characterized following previous publications (Dominici *et al.*, 2006). Briefly, SHED cell pellets were rinsed with PBS then centrifuged, harvested, and redispersed in PBS again. The harvested cells were incubated in 100 µl of PBS containing an antibody cocktail of CD45-APC (BioLegend, USA), CD90-FITC (BioLegend, USA), CD105-APC-A750 (BioLegend, USA) and CD73-PE (BioLegend, USA) for 30 minutes at room temperature. The cells were then washed with PBS and analyzed using a MACSQuant® VYB flow cytometer (Miltenyi Biotec, USA). The results were evaluated with MACSQuantify™ Software (Miltenyi Biotec, USA).

Differentiation assay of cultured SHED

For osteogenic differentiation, SHED were cultivated for 14 days in the OsteoDiff medium (StemMACS, USA), with the medium replaced every 3 days. Following differentiation, the cells were rinsed twice with PBS, fixed with 4% formaldehyde for 15 minutes, and then stained with Alizarin red (Sigma-Aldrich, USA) for 10 minutes to assess calcium deposition.

For adipogenic differentiation, SHED were incubated in AdipoDiff medium (StemMACS, USA) for 14 days, the medium was changed every 3 days. Upon completion of differentiation, the cells were rinsed twice with PBS, fixed in 4% formaldehyde for 15 minutes, and stained with Oil Red O (Sigma-Aldrich, USA) for 60 minutes to visualize lipid vacuole formation.

Cell viability assay

SHED in P3 were used as the starting materials. Initial cells were washed with PBS and then retrieved using 0.25% trypsin-EDTA and resuspended at a concentration of 10^6 cells/mL. For the hypothermic preservation study, SHED were maintained in LR (B Braun, Vietnam) or D5LR solution (Fresenius Kabi, Vietnam). These cells were analyzed after 2, 4, 6 hours at 4°C and 25°C. At the end of each time point, a cell viability assay was conducted using the trypan blue (Sigma-Aldrich, USA) following the manufacturer's guidelines. Control samples consisted of fresh cells that did not undergo any preservation and were measured at time 0 hour.

Cell adhesion assay

The cell adhesion was determined following a previous study (Chen *et al.*, 2013). Generally, after 6 hours of storage, suspensions of 2×10^4 cells in 100 µL of culture medium were seeded in each well of a 96-well plate and incubated for 24 hours at 37°C with 5% CO_2. Then, the plates were rinsed twice with PBS, fixed with 4% formaldehyde for 15 minutes, and stained with 1% crystal violet for 15 minutes. The post-storage SHED adhesion was measured by Multiskan™ FC Microplate Photometer (Thermo Scientific, USA) at a maximum wavelength of 550 nm.

Population doubling time

Following 6 hours of maintenance in storage solutions, SHED were seeded in each well of 24-well plates (Corning, USA) at a density of 10^4 cells/well with the first passage left for the recovery of cells. After that, the cells were cultured to reach approximately 80% confluency for the following 3 passages. Cells were harvested and the population doubling time (PDT) was evaluated as described in the previous study, using the following formula:

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

In which, t represents the total culture time, N1 denotes the initial cell number, and N2 represents the cell number at harvest.

Statistical analysis

The results were presented as mean ± standard deviation (SD) of the mean. One-way and two-way ANOVA were used to evaluate the data, and Dunnett's multiple comparisons were performed after each analysis. Statistical significance was accepted as $p < 0.05$.

RESULTS

Characterization of cultured SHED

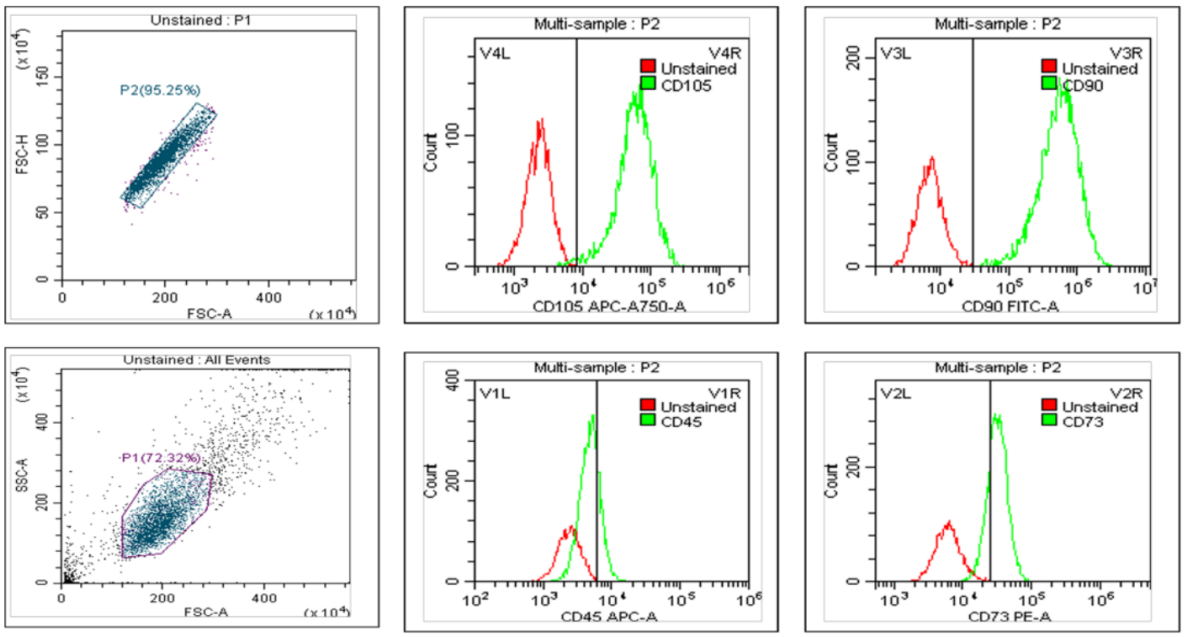

Figure 1. Phenotypic characterization of SHED was performed using flow cytometry analysis. The histograms demonstrate a low expression of the hematopoietic marker CD45 and a high expression of mesenchymal stem cell markers CD90, CD73 and CD105.

The identification and functional evaluation of SHED were performed using immunophenotyping and differentiation assays. Figure 1 demonstrates that SHED

lack the expression of the hematopoietic marker CD45, confirming their non-hematopoietic nature. In contrast, they exhibited high levels of MSC-specific markers, including CD90, CD105, and CD73, which align with the established characteristics of MSCs. The consistent detection of these markers across multiple samples underscores the reliability of the applied isolation and culture conditions.

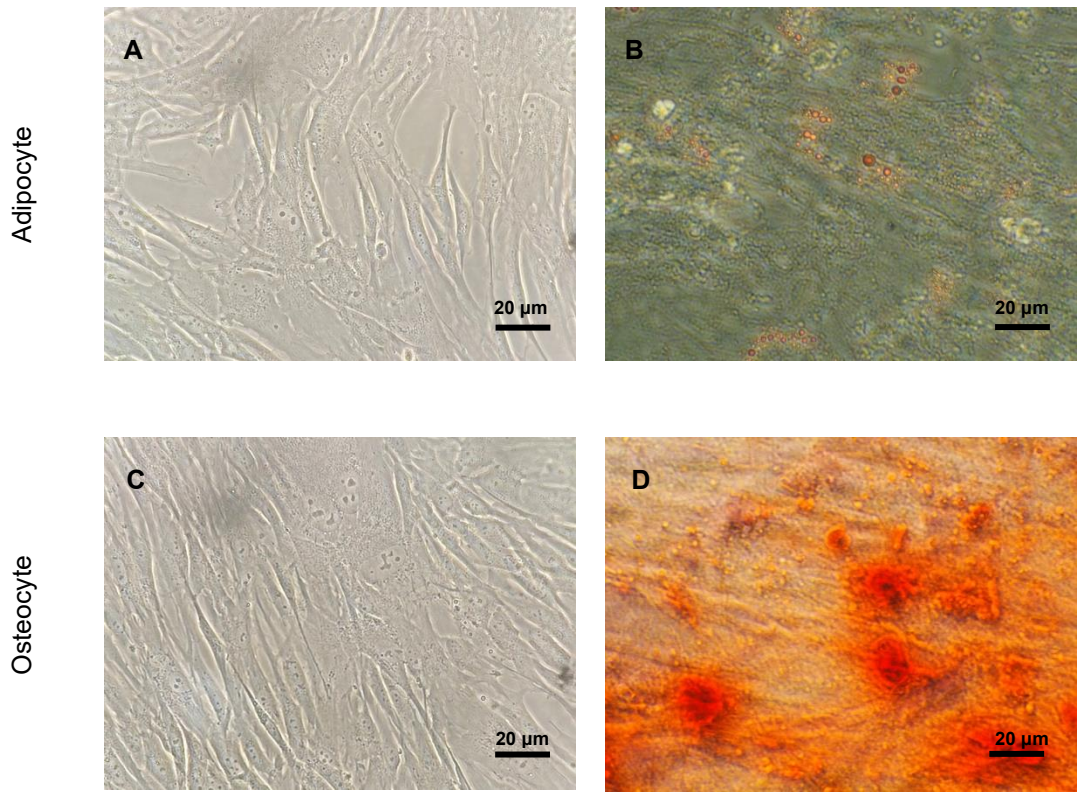

Figure 2. Adipogenic and osteogenic differentiation of SHED. Pre- (A) and mature adipocytes (B) were identified using Oil Red O staining, while pre- (C) and mature osteocytes (D) were detected with Alizarin Red staining. All samples were observed under a light microscope (40x).

Additionally, SHED showed remarkable potential for differentiation in multiple cell types. When subjected to adipogenic induction, SHED developed lipid vacuoles, as revealed by Oil Red O staining, indicating successful adipogenic differentiation (Figures 2A and B). Under osteogenic conditions, SHED exhibited mineralized matrix formation, confirmed by Alizarin Red staining, which highlighted substantial calcium deposition (Figures 2C and D). These observations demonstrate SHED's ability to differentiate into both adipogenic and osteogenic lineages, validating their multipotent capabilities.

Cell viability of post-storage SHED

Cell viability is a critical determinant for successful cell transplantation, with the FDA requiring a minimum survival rate of 70%

for transplanted cells. As shown in Figure 3A, SHED stored in LR at 4°C maintained a viability of 72.83 ± 5.07% after 6 hours, exceeding the FDA's threshold and demonstrating suitability for transplantation. However, at room temperature, SHED viability in LR declined more rapidly, starting at 70.49 ± 8.26% at 2 hours, decreasing to 63.22 ± 5.99% at 4 hours, and ultimately dropping to 53.53 ± 7.64% after 6 hours. These results indicate that lower temperatures are more effective in preserving cell viability when using LR.

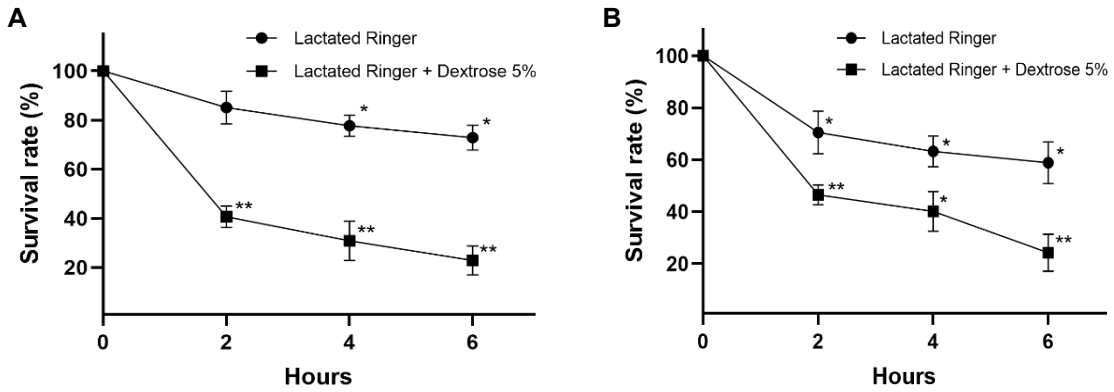

Figure 3. SHED viability under different preservation conditions over a 6-hour period. Viability rates were measured at 4°C (A) and room temperature (B) at 2, 4, and 6 hours. Data are presented as mean ± SD (n = 3 per condition), with significant differences from the initial value (time 0) denoted by *p < 0.05 and **p < 0.01, analyzed using two-way ANOVA followed by Dunnett multiple comparisons.

In comparison, SHED stored in D5LR exhibited a marked reduction in survival rates under both storage conditions. At 4°C, viability dropped significantly to 22.92 ± 5.9%, and at room temperature, it fell to 24.21 ± 7.13% within the same 6-hour period (Figures 3A and B). These findings reveal the adverse impact of high osmolarity in D5LR on SHED viability, particularly when compared to LR, which demonstrated superior preservation capacity under hypothermic conditions.

Cell adhesion of post-storage SHED

Cell adhesion is a crucial determinant of cell quality for transplantation, directly influencing survival, functionality, tissue integration, and overall therapeutic outcomes (Ahmad Khalili & Ahmad, 2015; Chen *et al.*, 2013). The impact of hypothermic preservation on SHED adhesion was assessed, revealing significant reductions compared to the control group, as illustrated in Figure 4. SHED stored in LR exhibited relatively stable adhesion levels under both temperature conditions, with adhesion rates of 65.11 ± 4.15% at 4°C and 63.48 ± 4.92% at 25°C. These findings suggest that LR provides an isotonic environment that supports partial retention of critical cell-surface proteins necessary for adhesion.

In contrast, SHED stored in D5LR displayed a pronounced decrease in adhesion capacity, with rates dropping to 5 ± 1.54% at 4°C and 2.28 ± 0.76% at 25°C. This substantial decline highlights the detrimental effects of D5LR's hyperosmotic nature, which likely disrupts integrin expression and interactions

with the extracellular matrix, key factors required for cell attachment and functionality.

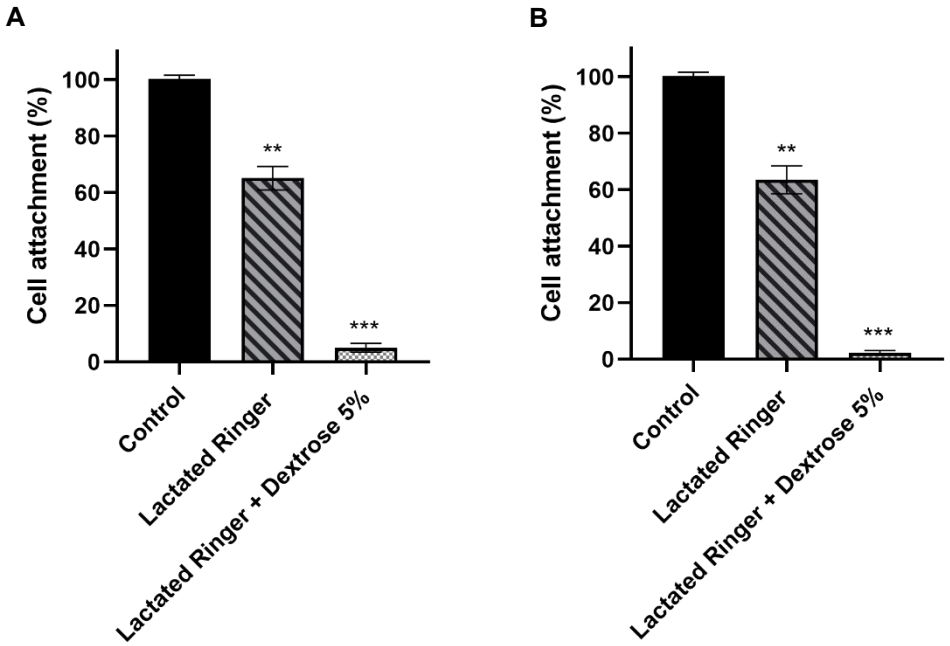

Figure 4. Assessment of SHED adhesion following suspension in various intravenous solutions for 6 hours at 4°C (A) and room temperature (B). Data are represented as mean ± SD (n = 3 per condition), with significant differences compared to the control sample indicated by **p < 0.01 and ***p < 0.001, analyzed using one-way ANOVA followed by Dunnett multiple comparisons.

Population doubling time of post-storage SHED

The effect of different post-storage conditions on the proliferative capacity of SHED was assessed using population doubling time analysis, as shown in Figure 5. SHED preserved in LR at both 4°C and 25°C maintained stable proliferation rates, with no significant differences compared to the control group. This stability indicates that LR effectively supports the retention of SHED's proliferative potential during short-term storage under these temperature conditions, making it a reliable preservation medium.

In contrast, SHED stored in D5LR solution exhibited a dramatic decline in proliferation. These cells showed a complete loss of proliferative capacity, evidenced by the absence of measurable population doubling over time. The significant reduction in PDT highlights the adverse effects of D5LR's composition on SHED viability and proliferation. While D5LR may have applications in other contexts, its inability to preserve the proliferative capacity of SHED renders it unsuitable for therapeutic scenarios requiring robust cell expansion.

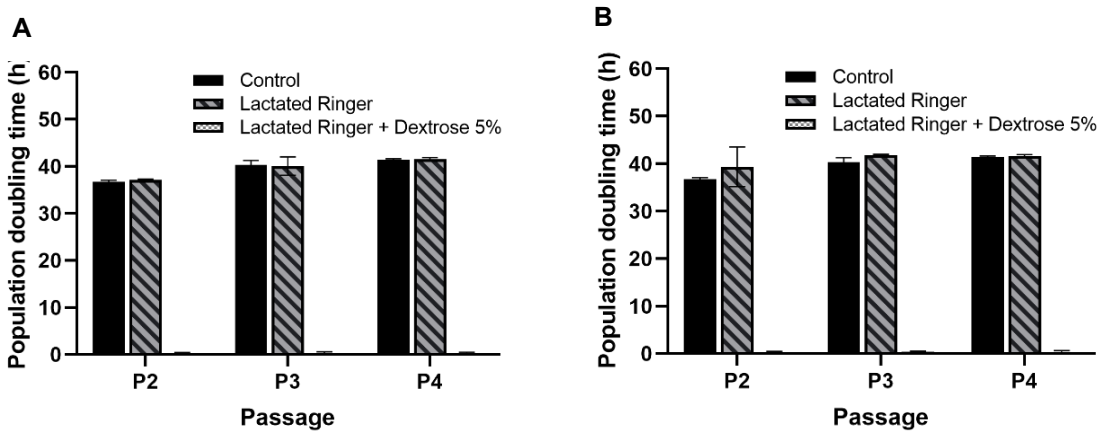

Figure 5. Evaluation of post-storage growth potential of SHED after suspension in various solutions for 6 hours at 4°C (A) and room temperature (B) over three consecutive passages. Data are represented as mean ± SD (n = 3 per condition), with significant differences compared to the control sample indicated by *p < 0.05, analyzed using two-way ANOVA followed by Dunnett multiple comparisons.

DISCUSSION

In this study, SHED cells were harvested from a Vietnamese donor and underwent consistent expansion, retaining their characteristic surface markers and differentiation potential in adipocytes and osteocytes. To our knowledge, this represents the first study utilizing SHED derived from a Vietnamese donor for such investigations. The results showed that LR at 4°C for 6 hours maintained a survival rate exceeding 70%, meeting FDA standards for cell transplantation. Additionally, SHED stored in LR preserved their proliferation capacity; however, a significant reduction in cell attachment was observed. Conversely, SHED stored in D5LR demonstrated a complete loss of survival, proliferation, and attachment capabilities.

MSCs are identified by their "stemness" markers and surface epitopes, which distinguish them from differentiated cells. However, unlike hematopoietic stem cells, which are well characterized by markers like CD34 used for immunoselection, a universal marker for MSCs has not been defined. This challenge is particularly pronounced when characterizing adipose-derived MSCs. Adipose stromal vascular cells can rapidly adopt a mesenchymal phenotype *in vitro*, and stromal cells are intricately organized around small blood vessels, further complicating their characterization (Ahmad Khalili & Ahmad, 2015). Additionally, variations in marker expression can arise due to differences in isolation methods, underscoring the need for standardization (Mushahary *et al.*, 2018). To provide clarity, the International Society for Cell & Gene Therapy (ISCT) established criteria defining MSCs. According to these guidelines, MSCs must adhere to plastic in culture, express CD105, CD73, and CD90, and lack the expression of CD45, CD34, CD14, CD11b, CD79a, CD19, and HLA-DR (Malbrain *et al.*, 2020). In the case of SHED, these cells have been shown to represent a

heterogeneous population of stem cells, as demonstrated through single-cell-derived colony analyses. SHED exhibit expression of embryonic stem cell markers, including Oct4 and Nanog, as well as keratan sulfate antigens Tra-1-81 and Tra-1-60 and stage-specific embryonic antigens SSEA-4 and SSEA-3 (Kerkis *et al.*, 2007). Interestingly, Pax6, the retinal stem cell marker, has also been detected in SHED, indicating their multipotency and diverse differentiation potential (Morsczeck *et al.*, 2008). Additionally, SHED express classical MSC markers such as CD105, CD90, and CD73, along with early cell surface markers like CD146 and STRO-1, which are also expressed in dental pulp stem cells, another type of dental mesenchymal stem cell (Gronthos *et al.*, 1999; Miura *et al.*, 2003). Our results indicated that variations in culture conditions did not have a significant effect on the expression of the surface markers CD105, CD73, and CD90. This consistency suggests that these markers are inherently stable and robust, highlighting their reliability as indicators for identifying and characterizing MSC populations, regardless of environmental or culture-related variables.

The ability of newly isolated adult stem cells to differentiate into multiple lineages is typically assessed to confirm their classification as MSCs. This involves evaluating their ability to differentiate into osteogenic and adipogenic lineages. SHED have demonstrated significant osteogenic potential, supported by various studies. When stimulated with an osteogenic cocktail containing dexamethasone, β-glycerophosphate or ascorbic acid, and retinoic acid, SHEDs upregulate both early osteogenic markers, such as runt-related transcription factor 2, alkaline phosphatase gene, and collagen type I alpha 1, as well as odontogenic differentiation and late osteogenic markers, including osteoprotegerin, osteopontin, osteocalcin, DMP-1, and DSPP (Chadipiralla *et al.*, 2010). Moreover, studies have shown that SHED have a significantly greater osteogenic differentiation capacity compared to human dental pulp stem cells and bone marrow mesenchymal stem cells (Nakajima *et al.*, 2018; Winning *et al.*, 2019). For example, gene expression analysis reveals that bone morphogenetic protein-4 (BMP-4) is expressed at markedly higher levels in SHED than in BMMSCs (Xu *et al.*, 2017). Furthermore, the osteogenic potential of SHED can be enhanced by factors like IL-17A, which promote mineralization activity (Sebastian *et al.*, 2018), and human β defensin 4, which enhances osteogenic differentiation under the influence of proinflammatory cytokines (Zhai *et al.*, 2019). SHED-incorporated carbonate apatite scaffolds were also found to enhance bone remodeling by upregulating BMP-2 and BMP-7 expressions while downregulating MMP-8 (Prahasanti *et al.*, 2020). On the other hand, their adipogenic differentiation has been studied using a specific induction process. SHED undergo adipogenic differentiation through a two-week incubation in an induction medium consisting of α-MEM enriched with indomethacin, human insulin, streptomycin, 3-isobutyl-L-methylxanthine, and dexamethasone. This is then followed by an additional week of culture in a maintenance medium, where α-MEM is supplemented with human insulin (Ko *et al.*, 2020). During this differentiation process, the morphology of SHED undergoes a notable transformation, changing from their original spindle-shaped form to a more polygonal shape, indicating successful progression

toward an adipogenic phenotype (Zhang *et al.*, 2016). The differentiation is further validated by the accumulation of intracellular lipids within the SHED, which can be visualized through Oil Red O staining. At the molecular level, adipogenically differentiated SHED exhibit significant upregulation of key adipogenic markers, including peroxisome proliferator-activated receptor-γ2 and lipoprotein lipase, as confirmed through reverse transcription polymerase chain reaction analysis (Miura *et al.*, 2003). The ability of SHED to undergo adipogenic differentiation adds to their classification as MSCs, offering potential insights for regenerative medicine and tissue engineering applications, even though their practical adipogenic use *in vivo* remains constrained. Consistently, our results indicated that the differentiation potential of cultured SHED in osteoblasts and adipocytes was not significantly influenced by the specific culture conditions employed in this study.

The preservation of cell viability during hypothermic storage is significantly influenced by the balance of soluble ions and their effects on cellular metabolism. Key contributors to hypothermia-induced injury include reduced enzyme activities and disruptions to metabolic and redox balances (Cosby *et al.*, 2008; Maathuis *et al.*, 2007). Hypothermic conditions impair the Na^+/K^+ ATPase, a critical ion transporter that maintains intracellular and extracellular Na^+ and K^+ concentrations. This loss of function leads to membrane depolarization and structural damage to the lipid bilayer and cytoskeleton (Bonventre & Cheung, 1985; Jamieson *et al.*, 1988). As a result, the plasma membrane becomes incapable of regulating ion fluxes, causing an imbalance of Na^+, K^+, Cl^-, Ca^{2+}, and Fe^{2+} ions (Guibert *et al.*, 2011). This disruption is often accompanied by water influx, leading to cell swelling and a decline in viability (Baicu & Taylor, 2002; Hochachka & Mommsen, 1983; Vreugdenhil *et al.*, 1999). In addition to monovalent ion imbalances, divalent ions such as Ca^{2+} play a significant role in cellular metabolism and structural stability. Hypothermic conditions elevate cytosolic Ca^{2+} levels, activating Ca^{2+}-dependent phospholipases and proteases, which further destabilize membranes and exacerbate cell swelling and necrosis. The accumulation of cytosolic Ca^{2+} also promotes the opening of mitochondrial permeability transition (MPT) pores, leading to mitochondrial membrane potential dissipation and accelerating cell death (Dai & Meng, 2011). This process is compounded by increased levels of cellular chelatable iron, which further promotes MPT pore opening and contributes to reactive oxygen species (ROS) overproduction (Alva *et al.*, 2013). Under normal physiological conditions, ROS are regulated by cellular antioxidant defense systems. However, hypothermic conditions impair these defenses, particularly by inhibiting enzymes responsible for catalyzing the decomposition of peroxide compounds. This loss of antioxidant activity exacerbates oxidative stress, ultimately leading to cellular apoptosis and necrosis. These injuries are interconnected, creating a self-reinforcing cycle of damage. For instance, oxidative stress caused by ROS can damage mitochondria, further disrupting metabolic processes and amplifying cellular injury. Additionally, one of the earliest impacts of hypothermia is the reduced activity of membrane ion pumps, such as sodium-potassium adenosine triphosphatase (Na^+/K^+ ATPase). Studies show that Na^+/K^+ ATPase operates at only 1% of its normal activity in

certain cell types at 5°C (Bates & MacKillop, 1985; Ellory & Willis, 1982). This decreased activity allows ions to diffuse across the membrane along concentration gradients, leading to ionic imbalances, osmotic cell swelling, and eventual cell death (Boutilier, 2001). A preservation medium with ion concentrations closely matching intracellular levels can mitigate this mechanism of injury. By maintaining proper sodium and potassium ion concentrations, the driving force for passive ion diffusion is eliminated, preventing ion flux when Na^+/K^+ ATPase activity is reduced. Our study demonstrated that LR provided superior preservation for SHED compared to D5LR at both storage temperatures. LR maintains optimal potassium and calcium concentrations, stabilizing the electrochemical gradient and preventing sodium influx, cell swelling, and lysis (Reddi, 2018). Sodium lactate buffers pH levels, reducing stress (Nuschke *et al.*, 2016). Conversely, D5LR's high osmolarity (525 mOsm/L) caused osmotic stress and cellular damage (Zhang *et al.*, 2022).

Cell adhesion in MSCs is a highly coordinated process influenced by factors such as the properties of the extracellular matrix (ECM) and the expression of specific adhesion molecules. This process is primarily mediated by integrins, including α1, α2, α3, α4, α5, αV, β1, β3, and β4, as well as other adhesion molecules such as VCAM-1, intercellular adhesion molecules, activated leukocyte cell adhesion molecule, and endoglin/CD105. Among these, integrin β1 plays a central role by facilitating MSC adhesion to ECM components like collagen, laminin, and fibronectin, enabling critical interactions between cells and their surrounding environment (Gronthos *et al.*, 1997; Lee *et al.*, 2004; Olivares-Navarrete *et al.*, 2015). The process of MSC adhesion occurs in distinct stages: initial attachment, spreading, actin cytoskeleton organization, and the formation of focal adhesions (FAs). Integrins are key mediators throughout these stages, forming patch-like distributions on the cell surface and connecting ECM proteins to intracellular cytoskeletal structures. These interactions ensure proper MSC orientation, spatial organization, and functional integration into tissues. However, insufficient ECM protein availability can disrupt integrin clustering and focal adhesion formation, impairing adhesion under physiological conditions (Farouz *et al.*, 2014; Massia & Hubbell, 1991). MSCs share similarities with leukocytes in their expression of adhesion molecules such as L-selectin, CD18, CD24, CD29, CD44, and CD49a–f, which facilitate interactions with the endothelium and other cells (Chamberlain *et al.*, 2007). Despite these shared features, MSCs utilize distinct adhesion mechanisms that are not yet fully understood. For instance, co-culture studies with endothelial cells indicate that MSC transmigration into tissues may employ some of the same adhesion molecules as leukocytes, though with specific differences in functionality (Chamberlain *et al.*, 2007; Couchman, 2010). Notably, MSC adhesion to the endothelium relies on interactions between P-selectin and its counter ligand, as well as VCAM-1 and its ligand, very late antigen-4, highlighting their critical roles in MSC homing and tissue integration (Segers *et al.*, 2006). The ability of MSCs to mobilize in response to physiological stimuli, such as skeletal muscle injury, acute burns, or chronic hypoxia, emphasizes the functional importance of adhesion molecules. Comparing adhesion molecule expression profiles between mobilized MSCs and tissue-resident MSCs offers

valuable insights into the mechanisms underlying MSC homing and migration (Ramírez *et al.*, 2006; Rochefort *et al.*, 2006). Furthermore, studies have observed variability in adhesion molecule expression across different cell culture passages, with some reporting significant changes while others suggest stable expression levels, underscoring the dynamic nature of MSC adhesion (Aldridge *et al.*, 2012; Lo Surdo & Bauer, 2012). In our study, cell attachment significantly declined after 6 hours of storage in LR, while cells in D5LR completely lost adhesion. Higher temperatures further reduced attachment. Previous studies showed MSCs stored at 2 to 8°C in solutions like 0.9% sodium chloride or Plasma-Lyte A maintained higher attachment rates than those stored at room temperature, likely due to slower ATP depletion and better preservation of integrins (Chen *et al.*, 2013).

Proliferative capacity is a critical factor in the success of MSC transplantation, as it ensures a sufficient number of cells to support tissue repair, regeneration, and immunomodulation. A reduction in this capacity can significantly compromise the therapeutic outcomes of MSC-based treatments, limiting their effectiveness in clinical applications (Petrenko *et al.*, 2019). Proliferation is particularly sensitive to environmental conditions, including hypothermia, which has been shown to inhibit cell division across various cell types, such as embryonic cells, somatic cells, and cancer cells (Carmeliet *et al.*, 1998; Eliasson & Jönsson, 2010; Eliasson *et al.*, 2010; Gardner *et al.*, 2001; Goda *et al.*, 2003). Under hypothermic conditions, DNA replication decreases significantly, as evidenced by reduced bromodeoxyuridine incorporation, a measure of DNA synthesis. This decline is especially pronounced in cells from wild-type models exposed to hypoxia. However, cells deficient in hypoxia-inducible factor 1-alpha (HIF-1α) do not exhibit the same reduction, indicating that the inhibition of proliferation under hypoxic conditions is critically dependent on HIF-1α activity (Carmeliet *et al.*, 1998; Goda *et al.*, 2003). This finding underscores the central role of HIF-1α in regulating the cellular response to hypothermia and hypoxia. Further research has demonstrated that the overexpression of HIF-1α alone is sufficient to induce cell cycle arrest, even in the absence of hypoxic stress. This highlights HIF-1α's potent regulatory influence on cell cycle progression and its ability to act as a key molecular switch during environmental stress (Hackenbeck *et al.*, 2009; Hubbi *et al.*, 2013). Our investigation revealed that SHED stored in LR retained proliferation rates comparable to the controls. In contrast, D5LR caused irreversible damage, eliminating proliferation (Liang *et al.*, 2012). Previous studies corroborated that umbilical cord MSCs stored in solutions like 5% dextrose or Plasma-Lyte A retained their proliferative potential. Similarly, LR preserved the proliferative ability of adipose-derived MSCs (Liang *et al.*, 2012).

CONCLUSION

In our investigation, SHED cells stored in LR solution at 4°C for 6 hours maintained high cell viability, adhesion, and proliferation capacity. In contrast, storage in the D5LR solution markedly impaired the post-storage functional properties of SHED. This study is the first to report the successful isolation of SHED from dental tissues of Vietnamese. Additionally, these results underscore the critical importance of

optimizing storage conditions to preserve cell quality during transportation and storage, which is pivotal for the success of cell-based regenerative therapies. The superior preservation of SHED in LR at 4°C highlights its potential as an optimal storage medium for stem cell applications. Further studies aim to refine preservation solutions and protocols to enhance the viability and functional integrity of SHED cells under hypothermic storage conditions, thereby advancing their clinical applicability in regenerative medicine.

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AUTHOR CONTRIBUTIONS

TSN: Review & editing, Supervision, Funding acquisition. TBCH: Investigation, Data curation, Writing-original draft. VTD: Conceptualization, Investigation, Review & editing, Supervision. TTNN: Investigation. TKHL: Investigation. DSN: Investigation. HHC: Review & editing, 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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