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Cryopreservation of Deciduous Teeth Originated Mesenchymal Stem Cells with Different Techniques

Süt Dişi Kaynaklı Mezenkimal Kök Hücrelerin Farklı Tekniklerle Kriyoprezervasyonu

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ABSTRACT

Objective: Mesenchymal stem cells (MSCs) are not used immediately after isolation in experimental and clinical studies. Traditional freezing and thawing processes may affect the cell viability or immunological properties of MSCs. In this study, the effect of different freezing methods on the viability, differentiation potential, and immunological properties of MSCs from human exfoliated deciduous teeth (SHED) was investigated.

Methods: SHED was isolated from SHED from healthy subjects aged between 8-12 years. The third passage cells were frozen with three different freezing solutions containing distinct concentrations of dimethyl sulfoxide, trehalose, ethylene glycol, or PVP40. After thawing, cells were analyzed for cell viability, co-cultured with mononuclear cells, and analyzed for lymphocyte proliferation rate, and cell viability for evaluating immunological properties.

Results: Compared to conventional cell freezing methods, the lymphocyte proliferation rate was suppressed, and cell viability ratios were increased with cryoprotectant solutions containing trehalose and ethylene glycol ($p<0.05$).

Conclusion: Cryoprotectant solutions containing trehalose, ethylene glycol, or PVP40 are more suitable for cell freezing.

Keywords: Mesenchymal stem cells, cryopreservation, cryoprotective agents

ÖZ

Amaç: Mezenkimal kök hücreler (MKH'ler) deneysel ve klinik çalışmalarda izolasyonlarından hemen sonra kullanılmamaktadır. Geleneksel dondurma ve çözme işlemleri, MKH'lerin hücre canlılığını veya immünolojik özelliklerini etkileyebilir. Bu çalışmada, farklı dondurma yöntemlerinin insan dökülen süt dişi (SHED) kaynaklı MKH'nin canlılık, farklılaşma potansiyeli ve immünolojik özellikleri üzerine etkisi araştırılmıştır.

Yöntemler: SHED kaynaklı MKH'ler, 8-12 yaş arası sağlıklı deneklerin SHED'lerinden izole edilmiştir. Üçüncü pasaja gelen hücreleri, farklı konsantrasyonlarda dimetil sülfoksit, trehaloz, etilen glikol veya PVP40 içeren üç farklı dondurma solüsyonu ile dondurulduu. Çözdürüldükten sonra hücreler, hücre canlılığı açısından analiz edildi ve mononükleer hücrelerle birlikte kültür edildi ve lenfosit çoğalma hızı ve immünolojik özelliklerin değerlendirilmesi için hücre canlılığı açısından analiz edildi.

Bulgular: Trehaloz ve etilen glikol içeren kriyoprotektan solüsyonlarıyla gerçekleştirilen dondurma yöntemleri konvansiyonel hücre dondurma yöntemlerine göre lenfosit proliferasyonu baskılanmış, hücre canlılığı oranları artmıştır ($p<0,05$).

Sonuç: Trehaloz, etilen glikol veya PVP40 içeren kriyoprotektan solüsyonlar hücre dondurma işlemi için daha uygundur.

Anahtar kelimeler: Mezenkimal kök hücreler, kriyoprezervasyon, kriyoprotektan ajanlar

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INTRODUCTION

Mesenchymal stem cells (MSCs) can differentiate into multiple lineages under suitable conditions, adhere to plastic surfaces and have a fibroblast-like morphology (1). Studies showed that MSCs play a regulatory role in the immune system by suppressing T and B -cell inflammatory responses (2).

Stem cells from human exfoliated deciduous teeth (SHED) are defined as cells that have a high proliferation capacity, can colonize, and differentiate into various cell types (e.g. neural cells, adipocytes, odontoblasts). It has been shown that SHED is better in terms of proliferation and differentiation ability compared to other oral cavity-derived MSCs (3).

Stem cells are not used immediately after isolation in experimental and clinical studies. It was frozen under laboratory conditions and then thawed again. Additionally, the cold chain route is used to transfer stem cells from one place to another. In this way, it is also appropriate to use the MSCs after special freezing procedures (4). A wide variety of methods are used in the cryopreservation of cells. Particularly significant studies have been conducted on sperm and embryo freezing (5). Fetal development is monitored by thawing frozen embryos and placing them in the uterus of experimental animals (6). In this project, the effect of different freezing methods on the viability, differentiation potential, and immunological properties of SHED was investigated.

METHODS

Isolation and Culture of SHED

SHED was isolated as described before (7). Briefly, human exfoliated deciduous teeth without abscesses were collected from healthy subjects aged 8-12 years. The clinical ethical approval was provided from the Marmara University Faculty of Medicine Clinical Ethical Committee with the protocol number 09.2014.0003 (date: 26.01.2023). Tissues were cut into 1x1 mm pieces and enzymatically digested with 3 mg/mL collagenase type I (Thermofisher, US) in phosphate-buffered saline (PBS) (Sigma-Aldrich, Germany) at 37 °C for 45 minutes. After the incubation period cell pellet was washed twice with Dulbecco' Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, hereafter complete DMEM (cDMEM). Cells were cultured with cDMEM in T25 culture flasks at 37 °C in 5% CO_2 for 7-10 days until they reached 80-90% confluence. The isolated cells were trypsinized with 0.25% Trypsin EDTA solution and cultured until the third passage.

Peripheral Blood Mononuclear Cell (PBMC) Isolation From Healthy Individuals

10 mL of venous blood was collected from the healthy subjects into heparinized tubes and the blood was transferred to a 15 mL sterile falcon for PBMCs isolation. Blood samples were diluted with PBS in a 1:1 (v/v) ratio. The samples were centrifuged at 2000 rpm for 20 minutes. After centrifugation, the buffy-coat collected in the middle of the tubes was transferred into sterile Falcon tubes and washed twice with 5 mL PBS.

Co-cultures of SHED with PBMC

After the SHED was thawed, it was resuspended in culture media and spread at $5x10^4$ cells per well of 48-well plates. Plates were incubated at 37 °C for 2 days for confluence. PBMC were labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured with SHED in a 1:10 ratio (SHED: PBMC). PBMC was stimulated with anti-CD3 and anti-CD28 (CDmix) for specific T lymphocyte stimulation. Lymphocytes were analyzed via flow cytometry after 72 h of the incubation period. The lymphocytes were analyzed for proliferation and CD4 + CD25 + FoxP3 + T regulatory cell frequency to determine the immunomodulatory effect of SHED after the thawing process.

Flow Cytometry Analysis

Analyses were performed after each subculture and using the FACS Calibur flow cytometer. After cells were removed, cells were counted and approximately $1x10^6$ cells were homogenized in PBS, incubated by adding 10 µL of fluorescent isothiocyanate (FITC) and phycoerythrin (PE)-conjugated monoclonal antibodies specific to the identified cell surface markers and appropriate isotype controls in the dark for 15 min. Wash buffer (PBS containing 0.1% sodium azide) was added after incubation and centrifuged at 1200 rpm for 5 min. The prepared cell suspension was analyzed via a flow cytometer (FACS Calibur, BD Biosciences, US). The isolated cells were stained with positive cell surface markers for MSCs as follows: CD146 FITC, CD29 APC, CD105 PE, CD90 PE, and CD73 PE. Apart from these, the cells were stained with CD14 PE, CD34 FITC, CD45APC, and HLA-DR as negative markers. Cell proliferation analysis was performed by staining cells with CFSE. Apoptosis was analyzed by staining cells with Annexin-V (FITC) and propidium iodide (PI). CD4 + CD25 + FoxP3 + T regulatory cell frequency was analyzed by staining cells with a human-FoxP3 detection kit (BD Biosciences, US).

Analysis of the Differentiation Potential of SHED

Adipogenic differentiation: The third passage cells were seeded on type I collagen-coated coverslips and allowed to differentiate into adipogenic lineage with the stimulation of the adipogenic medium [0.5-mM isobutyl-methylxanthine (IBMX-Sigma-Aldich), adipogenic medium MEM (Invitrogen/GIBCO)], 10% FBS (Invitrogen/GIBCO), 0.5 mM isobutyl-methylxanthine (IBMX-Sigma-Aldich), 10 M dexamethasone (Sigma-Aldich, Fluka Chemie AG, Buchs, Switzerland), 10 ug/mL insulin (Invitrogen/GIBCO), 200 µM indomethacin (Sigma-Aldich), and 1% antibiotic/antimycotic (Invitrogen/GIBCO) for 28 days at 37 °C 5% CO_2 chamber. The medium was changed twice a week. The adipocytes and oil droplets were evaluated by staining the cells with hematoxylin-eosin and 0.5% oil red O in methanol.

Osteogenic differentiation: The third passage cells were seeded on type I collagen-coated coverslips and allowed to differentiate into osteogenic lineages with the stimulation of an osteogenic

differentiation medium (StemPro™, Thermofisher, US). Cells were incubated for 21 days in a 37 °C, 5% CO_2 chamber. The medium was changed twice a week. The osteocytes and intercellular calcium deposits were evaluated by staining the cells with Alizarin Red.

Freezing of SHED by Traditional Slow Freezing and Vitrification Methods

In the vitrification technique used for freezing SHED, a freezing solution containing dimethyl sulfoxide (DMSO) was prepared using 2M DMSO, 1M acetamide, 3M propylene glycol, and 15% human albumin serum. Twenty one million cells/mL were transferred into 1.2 mL cryovials with 200 microliters of vitrification solution. Cryotubes and straws were dipped directly into liquid nitrogen immediately after loading with cryoprotectant and cell suspension. In the dissolution process, the cryovials and straws were dipped directly into the 37 °C water bath and the contents were poured into the washing solution containing 0.5 molar sucrose. The cells were pelleted by centrifugation at 1,200 rpm for 10 minutes, the supernatant was isolated, distributed to flasks with fresh culture medium, and cultured at 37 °C in a 5% CO_2 atmosphere.

In the second method, 3 different freezing solutions were prepared. Solution A; solution containing 600 µDMEM +300 µL FBS +50 µL DMSO was used for $5x10^5$ SHED. Solution B: RPMI medium containing 31.5% ethylene glycol + 4% PVP40 + 0.36 M trehalose + 10% FBS + 1% PSA was used for $5x10^5$ SHED. Solution C: RPMI medium containing 20% ethylene glycol + 10% glycerol + 10% FBS + 1% PSA was used for $5x10^5$ SHED. The cryopreserved cells were kept at -80 °C for 3 weeks.

Thawing of SHED

The cryovials were removed from the liquid nitrogen tank, slowly shaken horizontally in the air, and immersed in a 37 °C water bath for 1-2 minutes until the solution was completely dissolved in the cryovials. The cryovial content was transferred into a DMEM thawing solution containing 0.1-M trehalose and 10% FBS and left for 3-4 min. Cells were isolated from the thawing solution and transferred to culture wells for co-culture studies with lymphocytes.

Venous Blood Collection from Healthy Individuals and Peripheral Blood Mononuclear Cell Isolation

10 mL of venous blood was collected from the subjects into heparinized tubes and the blood was transferred to 15 mL sterile falcons for mononuclear cells isolation and diluted 1/1 (v/v) with PBS. Tubes were centrifuged at 2000 rpm for 20 min for the isolation of mononuclear cells. The buffy-coat remaining in the middle of the tubes after centrifugation was collected. Cells ($5x10^5$) were suspended in 1 mL cell culture medium and included in the co-culture study.

Labeling of PBMCs with CFSE

18 mM CFSE was used for the proliferation assay of PBMC. CFSE-labeled cells were kept at +4 °C for 6 minutes. Cells were suspended in cRPMI (RPMI 1640 + 10% FBS + 1% penicillin streptomycin) medium and centrifuged at 2000 rpm for 5 min. After the washing steps, cells were suspended in 1 mL cRPMI.

SHED and PBMC Co-culture

After thawing, SHEDs were resuspended in culture media and transferred to $5x10^4$ cells per well of 48-well plates. Plates were cultured for 2 days at 37 °C in a 5% CO_2 incubator for cell adhesion.

At the end of 2 days, PBMCs isolated from the venous blood of healthy individuals and labeled with CFSE were transferred to the wells as $5x10^5$ cells in each well. Unstimulated and anti-CD2 + anti-CD3 + anti-CD28 (CDmix)-stimulated cultures of mononuclear cells in each group were performed. After the culture period, the proliferation analysis of lymphocytes was analyzed via flow cytometry.

Cells Viability Analysis of Lymphocytes

To investigate the effects of cryopreserved SHEDs on the apoptosis of lymphocyte cells in healthy individuals, total viable cells were analyzed via flow cytometry. Cultured cells were stained with Annexin V (5 µL) and PI (5 µL) for 15 min. Staining procedures were performed according to the manufacturer's protocol (BD Biosciences, USA).

The lower left quadrants were analyzed via flow cytometry for viable cell ratios.

Statistical Analysis

The statistical analysis was performed using the GraphPad Prism 8.0 version (GraphPad Software, Inc., CA, USA). Data were given as mean ± standard deviation (minimum-maximum). Comparison of more than two groups was done by One-Way ANOVA test. $P<0.05$ values were considered statistically significant.

RESULTS

Characterization of SHED

The third -passage SHED showed fibroblast-like colonies (Figure 1), and showed MSC characteristics in the third passage with the high expression of positive markers (CD90, CD73, CD29, CD105, CD146), and lack the expression of negative markers (Figure 2). SHED was differentiated into osteogenic, chondrogenic, and adipogenic lineages (Figure 3).

SHEDs Frozen with Cryoprotectant Solutions Increased Cell Viability Ratio of PBMC

The apoptosis ratio of PBMC was analyzed in the presence and absence of SHEDs, which were frozen and thawed with different cryoprotectant solutions. At the end of the culture period, mononuclear cells were stained with Annexin V/PI and analyzed via flow cytometry for the cell survival ratio.

In the presence of SHEDs that were frozen and thawed with solution A, the cell viability ratio (72±5.6%) increased significantly compared to the culture group without SHED (22±4.7%) ($p<0.001$). In the presence of SHEDs that were frozen and thawed

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with solution B, the viability ratio (68±3.9%) increased significantly compared to the culture group without SHED (22±4.7%) ($p<0.001$). In the presence of SHEDs frozen and thawed with solution C, the viability ratio (71±4.4%) increased significantly compared to the culture group without SHED (22±4.7%) ($p<0.001$) (Figure 4).

SHEDs Frozen with Cryoprotectant Solutions Suppressed Lymphocyte Proliferation

Cell proliferation assays were performed with PBMCs isolated from peripheral venous blood from healthy individuals in the presence and absence of cryopreserved SHEDs. PBMC was co-cultured in the presence and absence of frozen and thawed SHED using

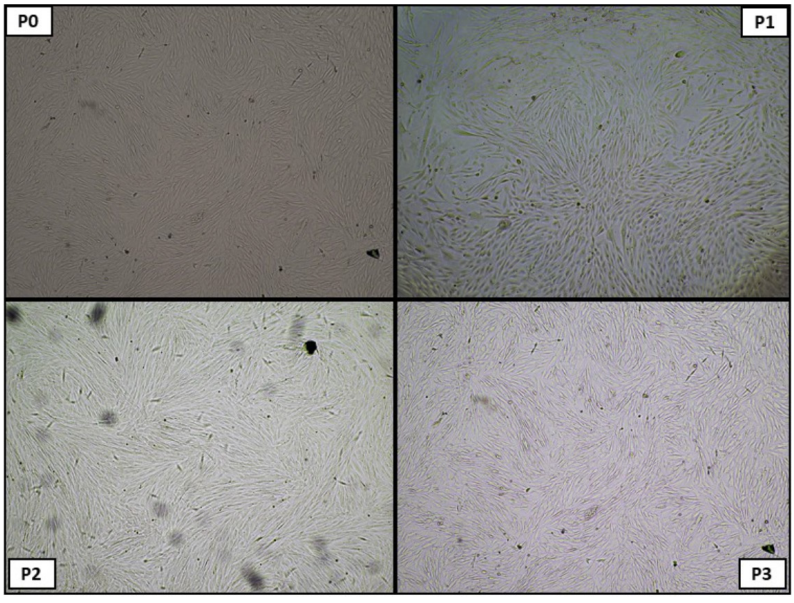

Figure 1. Morphology of SHEDs viewed under an inverted microscope. Fibroblast-like colonies appear in P0, P1, P2, and P3. P0; passage 0, P1; passage 1, P2; passage 2, and P3; passage 3

SHED: stem cells from human exfoliated deciduous teeth

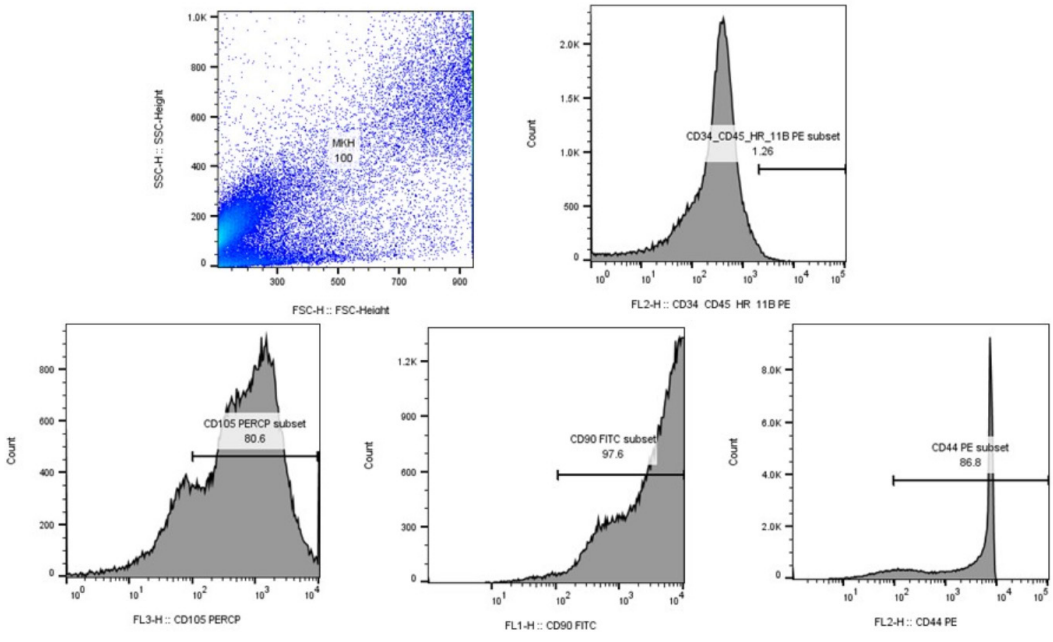

Figure 2. Immunophenotypic characterization of SHEDs. SHEDs in the third passage express positive markers CD105 (80.6%), CD90 (97.6%), CD44 (86.8%) specific to MSCs, and lack the expression of negative markers (CD45, CD34, CD11b, CD19, HLA-DR)

SHED: stem cells from human exfoliated deciduous teeth, MSCs: mesenchymal stem cells

three different techniques, and cell proliferation was analyzed on the 3rd day after culture. In the presence of SHEDs that were frozen and thawed with solution A, the proliferation rate (18±4.1%) was significantly reduced compared to the PBMC cultures alone (48±7%) ($p<0.005$). In the presence of SHED cells that were frozen and thawed with solution B, the proliferation rate (23±3.2%) was significantly reduced compared to the PBMC cultures alone (48±7%) ($p<0.005$). In the presence of SHEDs that were frozen and thawed with solution C, the proliferation rate (21±3.5%) was significantly reduced compared to the PBMC cultures alone (48±7%) ($p<0.005$). The suppression of the proliferation ratio of SHEDs frozen with three cryoprotectant solutions was similar, and there was no significant difference between the groups ($p>0.05$) (Figure 5).

DISCUSSION

The routine procedure used in cell freezing usually involves slow cooling in the presence of a cryoprotectant to prevent the damaging effects of intracellular ice formation. The use of the most cryoprotectant is DMSO and includes a controlled freezing technique at 1 to 2 °C/min and rapid thawing are considered standard (6). While current cryopreservation protocols are clinically effective, there are still about whether to whether they are optimal. DMSO is toxic to tissues and cells, toxicity depends

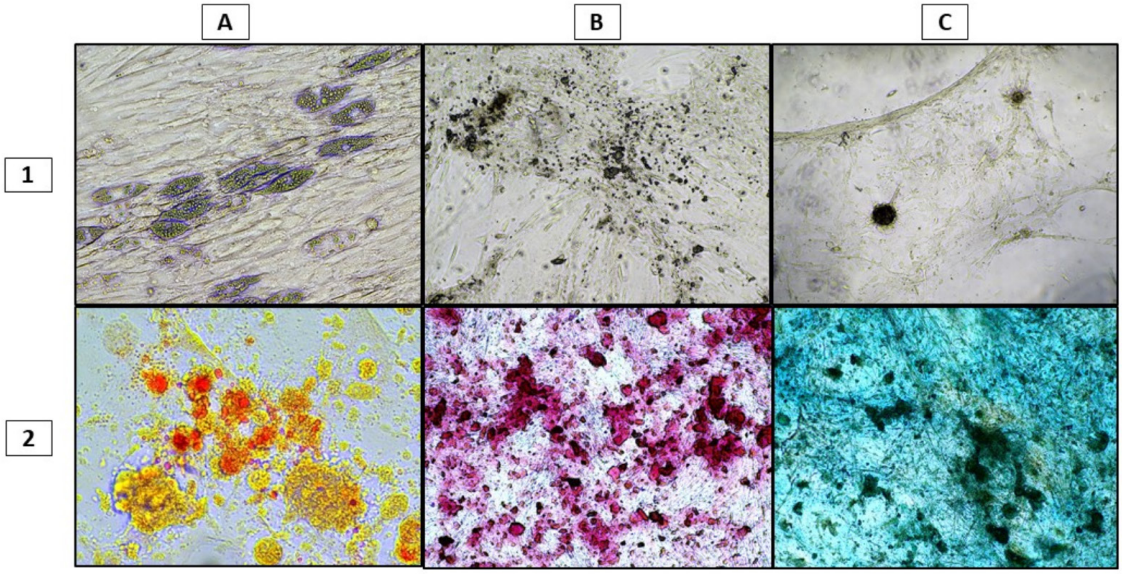

Figure 3. Adipogenic, osteogenic, and chondrogenic differentiation potential of SHEDs. The light microscope images of SHEDs in the third passage. **A)** Adipogenic differentiation of SHEDs. A1 shows inverted microscope image for colony morphology after adipogenic differentiation culture period. Oil droplets are stained orange with Oil red O stain in A2. **B)** Osteogenic differentiation of SHEDs. Osteocyte like colonies are shown in B1 under an inverted microscope. Calcium deposits are stained as red with Alizarin red stain in the intracellular matrix in B2. **C)** Chondrogenic differentiation of SHEDs. Condrocytes and cartilage formation was shown in C1 with a light microscope. Proteoglycans are stained blue with Alcian blue stain in C2

SHED: stem cells from human exfoliated deciduous teeth

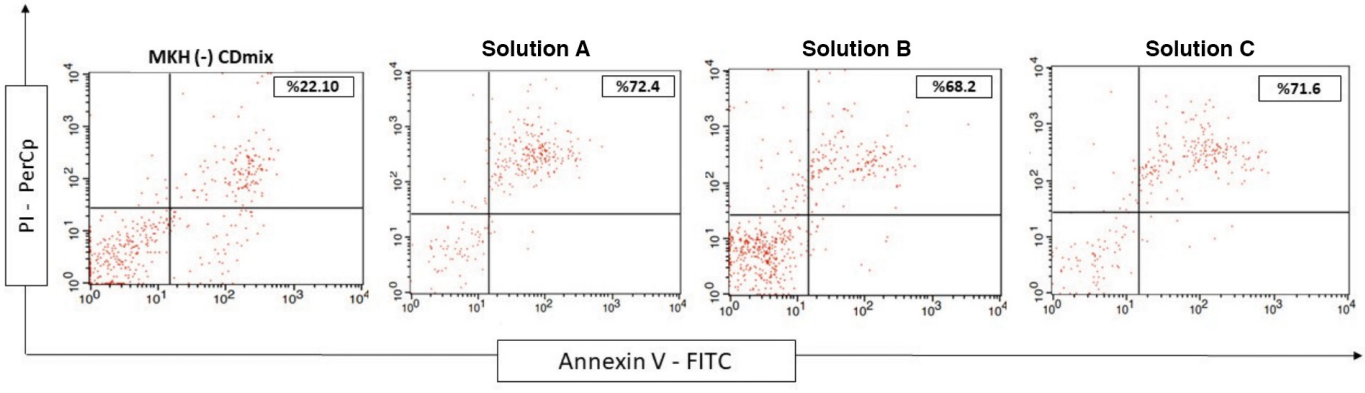

Figure 4. Cell viability analysis of SHEDs. The viability ratio of SHEDs is high after freezing and thawing with cryoprotectant solutions

SHED: stem cells from human exfoliated deciduous teeth

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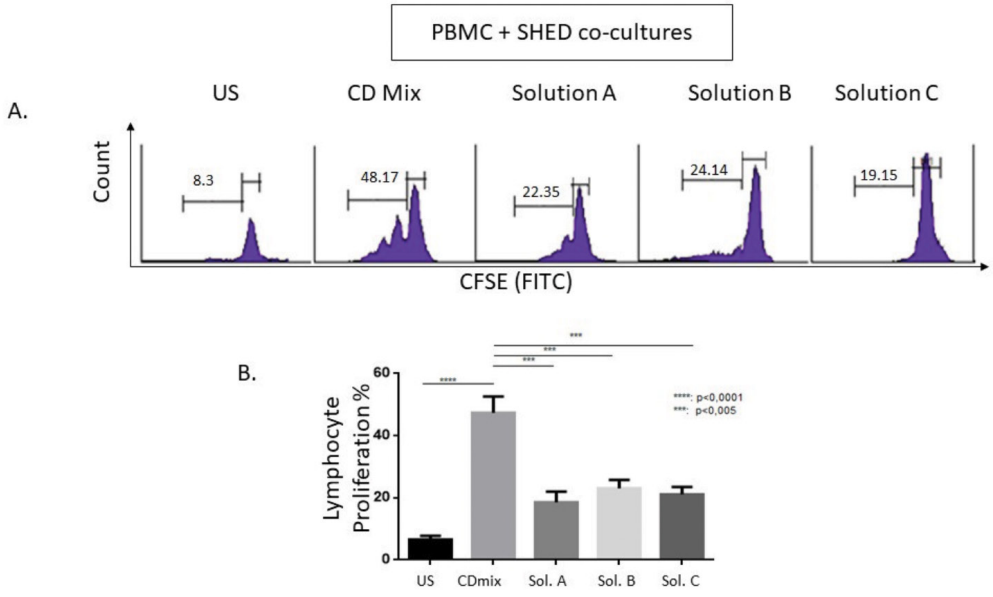

Figure 5. Flow cytometry analysis for lymphocyte proliferation rate. After the co-culture of SHEDs with PBMC, PBMC was analyzed for proliferative responses. **A)** Flow cytometry analysis of lymphocytes after co-culture with cryopreserved SHEDs. **B)** Statistical analysis of lymphocyte proliferation rate after co-culture of lymphocytes with cryopreserved SHEDs. The data showed that cryoprotectant solutions (solution A, solution B, and solution C) can protect the immunologic properties of SHEDs. US; unstimulated PBMC, CDmix; stimulated lymphocytes

SHED: stem cells from human exfoliated deciduous teeth, PBMC: peripheral blood mononuclear cell

on time, temperature, and concentration. Toxicity varies with cell type and accepted practice is to use the cryoprotectant for a short time at low temperatures (+4 °C) in practical.

MSCs obtained from bone marrow and cord blood can be frozen by the methods used for cryopreservation of hematopoietic analogs and by slow freezing protocols using DMSO used in many studies (8,9). 10% DMSO and slow cooling/rapid heating do not affect the viability or differentiation potential of adipose tissue-derived MSCs (10). Adult MSCs from human dental pulp also showed high post-dissolution viability and three lineage differentiation after slow cooling in 1-1.5 mol/L DMSO (~7.5-10%), and it is superior to propylene glycol (11).

It is known that DMSO affects the epigenetic profile and differentiation induction of mouse stem cells (12). However, since it has side effects in its use as a cryoprotectant in hematopoietic cell therapy, in addition to cell toxicity, it has led to the use of freezing applications of MSCs with or without other cryoprotectants (13). Many alternative cryoprotectant formulations have been developed to eliminate animal serum from cryoprotectant solution, which are both cost-effective and suitable for clinical use, and for situations where the terminal sterilization is impossible. However, this is also expensive and poses a potential danger in transmitting human pathogens (14).

Polyvinylpyrrolidone (PVP) is an extracellular cryoprotectant, investigated as an alternative to cryopreservation with DMSO and FBS. The recovery and differentiation capacity of cells were studied after equilibration in a number of different cryopreservation media, 'dump' freezing to -80 °C and storage in liquid nitrogen. The recovery of cells cryopreserved in 10% PVP with human serum may be better than cells stored frozen in DMSO, although lower with animal serum. A similar study used methylcellulose alone or in conjunction with reduced DMSO levels and demonstrated that human serum can alter FBS in standard DMSO mixtures without affecting cell healing, with 1% methylcellulose yielding as low as 2% compared to DMSO concentrations (15). In a previous study, Annexin V was used to analyze cells 24 h after thawing for apoptosis analysis, and it was observed that adipogenic and osteogenic differentiation of these cells were preserved (16). Liu et al. (17) approved the use of reduced DMSO for good quality and non-differential (xeno-free) cryopreservation medium for bone marrow stem cells used in cellular therapy. They used polyethylene glycol (PEG) and trehalose with DMSO concentrations between 2.5% and 7.5% instead of FBS. Standard slow cooling with DMSO and 10% FBS methods were compared by measuring cellular viability, proliferative capacity, and differentiation potential. The results showed that the use of PEG with reduced DMSO concentrations instead of FBS was comparable, but only when 2% bovine serum albumin was used. Besides, 10% DMSO and 90% FBS gave better results than other combinations in cryopreservation. In contrast to cord blood, bone marrow, or peripheral blood-derived hematopoietic cell studies, trehalose is ineffective, but effective along with DMSO at a reduced rate (17).

In this study, we demonstrated that three different cryoprotectant solutions are ideal for freezing MSCs. It has been shown that these cryoprotectant solutions are non-toxic on SHED cells and are also

suitable for long-term storage in the freezer without changing the immunological properties of SHEDs.

With regard to the review of the literature on cryoprotective agents' biological response to human dental pulp stem cells, this study contributes to the knowledge regarding how these agents affect this process as part of the literature. A comparison of the use of PEG with reduced DMSO concentrations instead of FBS revealed that the results were comparable, but only when 2% bovine serum albumin was used instead of FBS. It has also been found that 10% DMSO and 90% FBS have been more effective in cryopreservation than other cryoprotectant combinations, compared with cord blood, bone marrow, or peripheral blood-derived hematopoietic cell studies, trehalose was shown to be ineffective. However, it was shown to be effective in a cryoprotectant combination with DMSO at a reduced rate, when used along with DMSO.

Study Limitations

The limitation of our study is the absence of a control group follow-up. Due to the limited number of cells that could be obtained from donor patients, no further experimental groups were formed as a result. Cells were not stored for more than a year in frozen form It was not possible to eliminate genetic variations since the dental stem cells were obtained from different patients.

CONCLUSION

To conclude, we have found that three different cryoprotectant solutions are ideal for freezing MSCs, among them liquid nitrogen. It has been shown that these cryoprotectant solutions are non-toxic on SHED cells and are also suitable for long-term storage in the freezer without changing the immunological properties of SHEDs.

Ethics Committee Approval: The clinical ethical approval was provided from the Marmara University Faculty of Medicine Clinical Ethical Committee with the protocol number 09.2014.0003 (date: 26.01.2023).

Informed Consent: Patient consent is not required for this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - T.A., Tu.A.; Design - T.A., D.G., Tu.A.; Data Collection and/or Processing - T.A., D.G., Tu.A.; Analysis and/or Interpretation - T.A., Tu.A.; Literature Search - T.A., D.G., Tu.A.; Writing - T.A., D.G., Tu.A.

Conflict of Interest: The authors have no conflict of interest to declare.

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