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Optimisation of cryopreservation conditions, including storage duration and revival methods, for the viability of human primary cells

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Abstract

Background Cryopreservation is a crucial procedure for safeguarding cells or other biological constructs, showcasing considerable potential for applications in tissue engineering and regenerative medicine.

Aims This study aimed to evaluate the effectiveness of different cryopreservation conditions on human cells viability.

Methods A set of cryopreserved data from Department of Tissue Engineering and Regenerative Medicine (DTERM) cell bank were analyse for cells attachment after 24 h being revived. The revived cells were analysed based on different cryopreservation conditions which includes cell types (skin keratinocytes and fibroblasts, respiratory epithelial, bone marrow mesenchymal stem cell (MSC); cryo mediums (FBS + 10% DMSO; commercial medium); storage durations (0 to > 24 months) and locations (tank 1–2; box 1–5), and revival methods (direct; indirect methods). Human dermal fibroblasts (HDF) were then cultured, cryopreserved in different cryo mediums (HPL + 10% DMSO; FBS + 10% DMSO; Cryostor) and stored for 1 and 3 months. The HDFs were revived using either direct or indirect method and cell number, viability and protein expression analysis were compared.

Results In the analysis cell cryopreserved data; fibroblast cells; FBS + 10% DMSO cryo medium; storage duration of 0–6 months; direct cell revival; storage in vapor phase of cryo tank; had the highest number of vials with optimal cell attachment after 24 h revived. HDFs cryopreserved in FBS + 10% DMSO for 1 and 3 months with both revival methods, showed optimal live cell numbers and viability above 80%, higher than other cryo medium groups. Morphologically, the fibroblasts were able to retain their phenotype with positive expression of Ki67 and Col-1. HDFs cryopreserved in FBS + 10% DMSO at 3 months showed significantly higher expression of Ki67 (97.3% ± 4.62) with the indirect revival method, while Col-1 expression (100%) was significantly higher at both 1 and 3 months compared to other groups.

Conclusion In conclusion, fibroblasts were able to retain their characteristics after various cryopreservation conditions with a slight decrease in viability that may be due to the thermal-cycling effect. However, further

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investigation on the longer cryopreservation periods should be conducted for other types of cells and cryo mediums to achieve optimal cryopreservation outcomes.

Highlights

- Cryopreservation optimizes cell recovery, viability for tissue engineering. Fibroblasts maintain phenotype in FBS + 10% DMSO, but longer periods need scrutiny.

Keywords Cell revival, Cryopreservation, Cryo medium, Fibroblast, Proliferation marker

Introduction

Cryopreservation involves preserving living cells and tissues at extremely low temperatures to maintain their structural integrity for long-term storage [1]. By cooling to ultra-low temperatures (below −130 °C) until extracellular ice forms, the process slows down biological aging by reducing the cells' kinetic energy and molecular motion [2, 3]. However, inducing hypothermia conditions without intervention results in cell dehydration and gradual cell damage during three phases: cooling, maintenance in the cold, and rewarming [4, 5]. Accumulated extracellular ice crystals can also cause osmotic shock and increased toxicity by trapping cells resulting in damage [6]. Thus, optimising the cooling rate and using cryoprotectants are key to reducing damage and improving cell survival. Under an efficient protocol, the freezing enhances longevity and confers stability in living cells. In addition, there is a great diversity in the cryobiological and cryo survival response of cells depending on their type of cells and biological species [7].

Cryoprotective agents reduce the freezing injury triggered by the cryopreservation process. These agents should be low in toxicity, able to enter cells, and safe for biological use. Depending on the cell type, cooling speed, and reheating rate, various cryoprotective chemicals have been developed to minimise ice crystallization formation and controlling process to protect the cells [8]. A simple preservation container, known as Mr. Frosty Freezing Container or 'CoolCell', provides an optimal cooling rate of about −1 °C per minute for preserving cells. To achieve the best cell and tissue viability, it's important to optimise factors such as sample amount, cooling and warming rates, and cryoprotectant concentration based on the specific type of cells and tissues [9].

Cryoprotectants are categorised into two types: membrane-permeating and non-membrane-permeating. Membrane-permeating agents, like dimethyl sulfoxide (DMSO), glycerol, and 1,2-propanediol, enter cells and lower electrolyte concentrations, protecting them from damage. Non -permeating agents, such as 2-methyl-2,4-pentanediol and polymers like polyvinyl pyrrolidone, hydroxyethyl starch, and certain sugars help prevent ice formation outside the cells [7]. DMSO is commonly used because it is affordable and has low cytotoxicity [10], making it suitable for various cell types. Human primary cells, often expensive and difficult to obtain, are challenging to grow and have limited culture passages. Therefore, cryopreservation of primary cells is important to preserve their unique characteristics and availability for future use in research and clinical applications. Synthetic cryo medium, being chemically defined and free from animal-derived components, offers consistency and safety, making it ideal for clinical applications [11]. However, optimisation of cryopreservation conditions is crucial for maintaining the viability of human primary cells.

This study aims is to evaluate cell performance under different cryopreservation conditions, such as various cell types, cryo medium, storage durations, storage locations, and cell revival methods, using data obtained from the DTERM primary cell bank, previously known as the Centre for Tissue Engineering and Regenerative Medicine (CTERM). Additionally, the viability and characteristics of revived HDF after cryopreservation in different cryo medium after 1 and 3 months were compared. This study is expected to provide new information on how primary human dermal fibroblast cells behave with different cryo medium after being cryopreserved. It aims to identify the best cryopreservation conditions that can potentially serve as guidelines to maximize the effectiveness of cryopreserved cells.

Materials and methodology

This study is conducted following UKM research ethics committee approval with the reference number: UKM PPI/111/8/JEP-2023-007. The cryopreserved cell for this study was obtained from the DTERM primary cell bank with ethics approval number: [UKM1.5.3.5/244/FF-2015-376] and patient consent.

Cell cryopreservation data collection

Several sets of cryopreserved cells were revived after being cryopreserved for different lengths of time (ranging from 0 to >24 months). This is to evaluate whether different cryopreservation conditions affect cell performance and viability. The first condition examined was the type of cells, which included: keratinocytes and fibroblasts from the skin, respiratory epithelium from nasal turbinate, and bone marrow mesenchymal stem cell (MSC). The second condition evaluated was the type of cryo medium, comparing a commercial medium from

the supplier with a mixture of Fetal Bovine Serum (FBS) (LSP, UK) and 10% DMSO (Sigma, Germany). Next was to evaluate the different storage locations. Cells were placed in different boxes within two tanks, each containing four racks and five boxes per rack. Boxes 4 and 5 were immersed in liquid nitrogen, while boxes 1, 2, and 3 were not, to determine if immersion in liquid nitrogen (liquid phase) affected cell viability and performance. The study also evaluated different storage durations by recording the cryopreservation date and comparing it to the duration of storage at the time of revival. During this revival, the duration of storage was taken and recorded. Cells were quickly thawed (<1 min) by gently swirling the vial in a 37 °C water bath. The final condition assessed was the method of cell revival, either by direct seeding or indirect seeding (after centrifugation). The performance of revived cells was inspected after 24 h by observing the percentage of cell attachment, which indicated cell viability and growth. Images of the 24 h cell inspection were recorded, and any contamination was noted. The percentage of cell attachment was based on the number of cells attached per area of the images over the initial cell number. It was categorized as 0–20%, 21–30%, 31–60%, or more than 60%. These data on cell performance were collected and analysed using GraphPad.

HDF revival and cryopreservation

HDF were counted for cell number and viability and characterised via immunochemistry staining for Ki-67 and collagen type 1 (Col-1). The cryopreserved cells were thawed and resuspended in F12:Dulbecco's Modified Eagle Medium (DMEM) medium (Sigma, USA) with 10% FBS (F12:DMEM+10% FBS) [12]. The cells were incubated at 37 °C in 5% CO_2 with the medium replaced every 2–3 days until reached 70–80% confluency. Cells were then sub-cultured to obtain a sufficient number for short-term cryopreservation experiments. The initial number of viable cells was counted, and the cells were suspended in different cryo mediums: FBS+10% DMSO, HPL+10% DMSO, and 5% CryoStor (Stemcell, Canada) (referred to as FBS, HPL, and CS respectively) [13, 14]. The cells were cryopreserved by transferring them into CoolCell freezing container (Corning, USA) and freezing at -80 °C for a minimum of 4 h before being transfer to a liquid nitrogen tank for stored at 1 and 3 months [1]. After 1 and 3 months, the cells were revived using two different methods: (a) the direct method, where cryopreserved cells were thawed, resuspended with a new fresh medium, and directly seeded for culture [15–17], or (b) the indirect method, which involved an additional centrifugation step (5000 rpm for 5 min) to remove the supernatant before cell seeding and culture [18, 19]. All cultures were observed for cell viability, growth and immunocytochemistry analysis [1, 20, 21]. Each group had three samples ($n=3$).

Fibroblast viability and cell growth

The viability of fibroblasts was assessed using 0.4% Trypan Blue dye (Sigma) and a hemocytometer. Dead cells (stained) and live cells (unstained) were counted [22].
Total number of cells = Cell concentration/mL x total volume of cell suspension (mL).
% Viability = (Lives cell counted/Total cell counted) x 100.

Immunocytochemistry staining and analysis

HDF were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for at least 15 minutes, permeabilised with 0.1% Triton X-100 solution (Sigma-Aldrich) for 20 min, and block with 10% goat serum (Capricon, Germany) for 1 h at 37°C. Then, the cells were incubated with primary antibody; mouse anti-Collagen Type 1 (Col-1) (Abcam, UK) and rabbit anti-human Ki67 antibody (Abcam) overnight at 4°C. On the next day, the cells were incubated with secondary antibody; goat anti-mouse IgG Alexa Fluor 488 (green)/594 (red) (Invitrogen, USA) for 2 h at 37°C. The cells then were counterstained with DAPI (4',6-diamidino-2-phenylindole) (Dako, Denmark) for 20 min at room temperature and observed using Nikon A1R confocal microscope (Nikon, Japan).

Statistical analysis

The quantitative data of total number of live cells, percentage of cell attachment (cell viability), and immunocytochemistry images are reported as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 10.0 (GraphPad Software, La Jolla, CA, USA) and the results were analysed using two-way analysis of variance (ANOVA). The difference between groups was significant if $p<0.05$.

Results

Cell cryopreservation data collection

Data on the percentage of cell attachment after 24 h for different types of human cells were collected from the DTERM cryopreservation data. The cells were inspected for 24 h, and attachment on the culture flask was categorised as 0–20%, 21–30%, 31–60%, or more than 60%. Figure 1A showed fibroblasts have the highest percentage of cells with more than 60% attachment and the highest with less than 20% attachment, likely due to widespread use in various studies. Figure 1B indicates that FBS with 10% DMSO resulted in the highest number of vials with more than 60% attachment compared to commercial mediums. Figure 1C shows that within 24 months storage duration, the cells are still viable and survive, with most vials having less than 20% attachment. The indirect method also resulted in the lowest attachment, with less

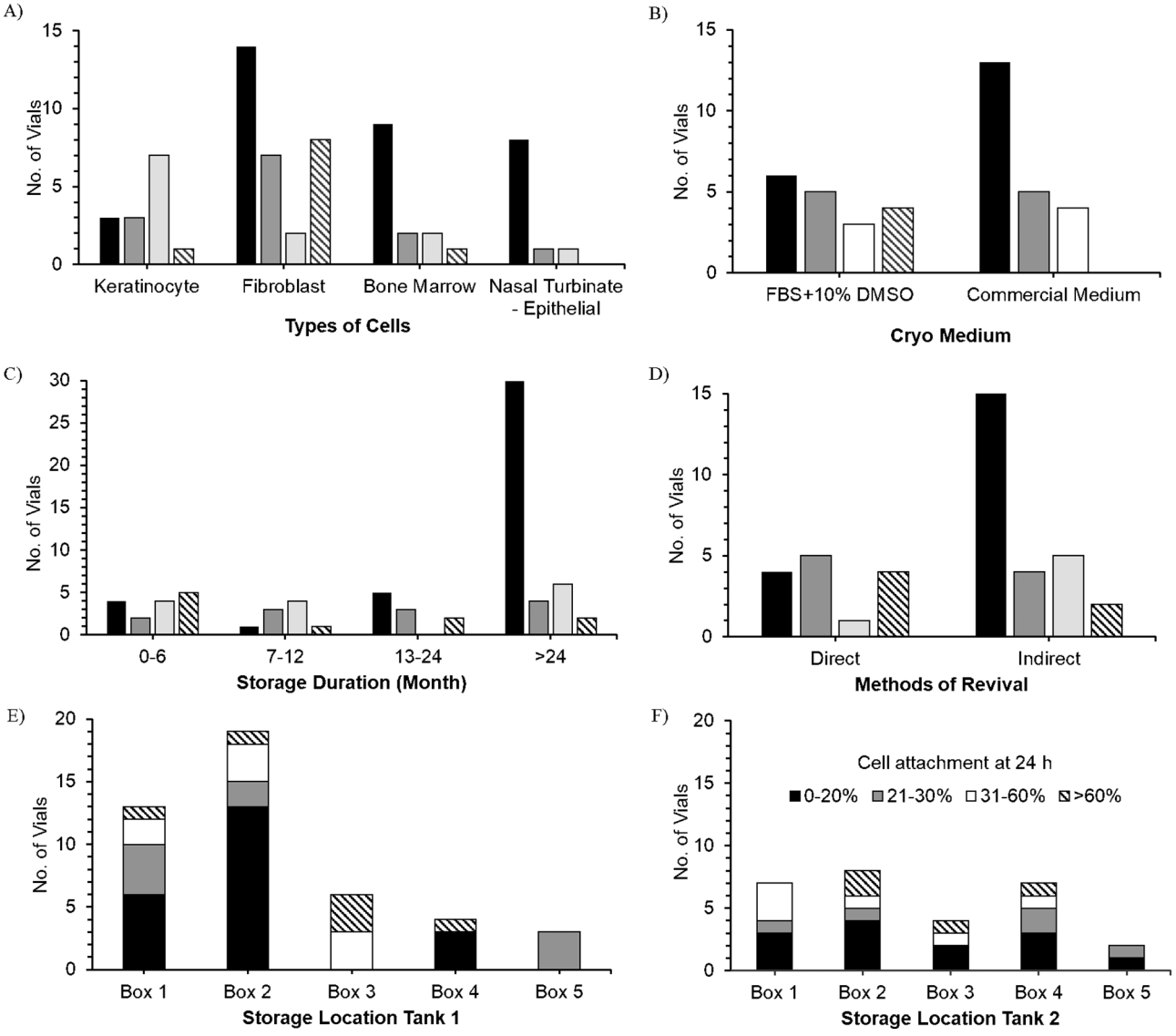

Fig. 1 The number of vials according to different storage conditions. The percentage of cell attachment after 24 h observed in (**A**) different types of cells, (**B**) different cryo mediums, (**C**) different storage durations, (**D**) different methods of revival, and (**E**) and (**F**) different storage locations in tank 1 and tank 2, respectively

than 20%, as shown in Fig. 1D. Figure 1E and F reveal that vials stored at the vapor-liquid level in boxes 3 and 2 achieved the best attachment rates, with more than 60% cell attachment after 24 h revived.

In-vitro analysis of viability of cryopreserved HDF

The total number and percentage of viability of HDF were assessed after 1 and 3 months of cryopreservation using different cryo mediums and revival methods (Fig. 2). For 1 month of cryopreservation, all groups showed a significant decrease in the number of live cells on day 0 after revival, ranging from 1.5×10^5 to 5.5×10^5 cells compared to before cryopreservation which is 1×10^6 cells (Fig. 2A). However, on day 7 after revival, FBS+10% DMSO with both revival methods showed the highest number of live cells, increasing by 0.5×10^5 to 1.0×10^5 cells compared to day 0. For 3 months of cryopreservation, all groups also showed a significant decrease in the number of live cells on day 0 after revival, ranging from 1.0×10^5 to 4.5×10^5 cells, except for FBS+10% DMSO with the direct method, which only decreased by 0.5×10^5 cells (Fig. 2B). On day 7, FBS+10% DMSO with both revival methods had the highest numbers of live cells, increasing by 0.3×10^5 to 0.5×10^5cells compared to day 0.

As for the cell viability, for 1 month of cryopreservation, all cryo medium groups showed a significant decrease in viability on day 0 after revival, ranging from 10 to 45%. However, on day 7, FBS+10% DMSO with the direct

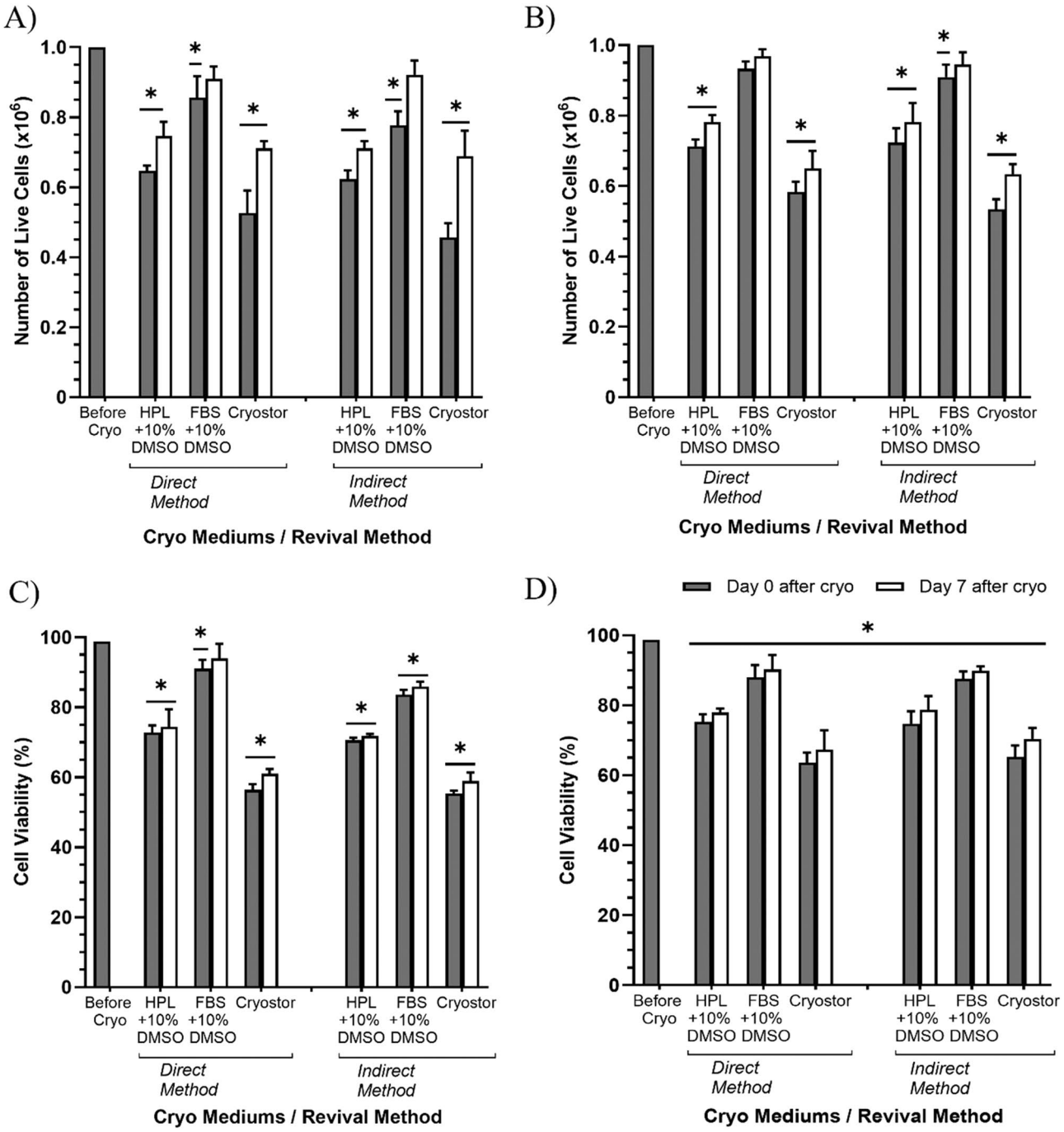

Fig. 2 Number of cells and percentage of viability after cryopreservation for 1 and 3 months. (**A**) Number of cells for 1 month, (**B**) Number of cells for 3 months, (**C**) Percentage of cell viability for 1 month, and (**D**) Percentage of cell viability for 3 months. ***** Represents a significant difference compared to before cryopreservation with $p < 0.05$

revival methods had the highest viability, increasing by 5% compared to day 0 (Fig. 2C). For 3 months of cryopreservation, all groups showed a significant decrease in viability on day 0, ranging from 10 to 35%. On day 7, there was a slight increase in viability for all groups, ranging from 3 −7% as compared to day 0 (Fig. 2D).

Cell morphology

In terms of cell morphology, images were taken on day 1, 3, and 7 after the revival of cryopreserved cells from three different cryo mediums and two revival methods (direct and indirect). Fibroblasts exhibit an elongated, spindle-shaped morphology for both 1 month (Figs. 3) and 3 months (Fig. 4) of cryopreservation. This

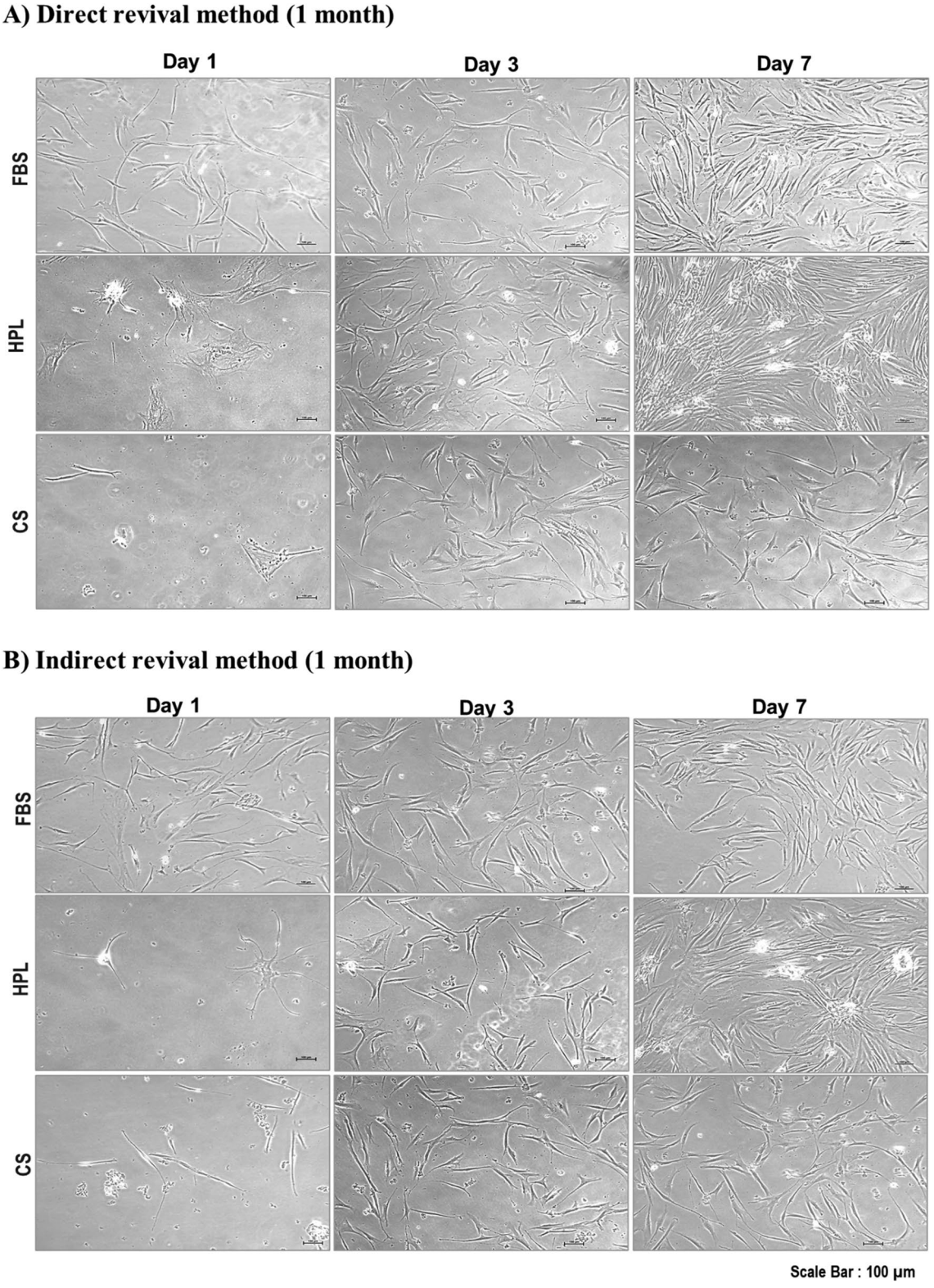

Fig. 3 Morphology of HDF after 1 month of cryopreservation using (**A**) direct and (**B**) indirect revival methods. The scale bar is 100 µm

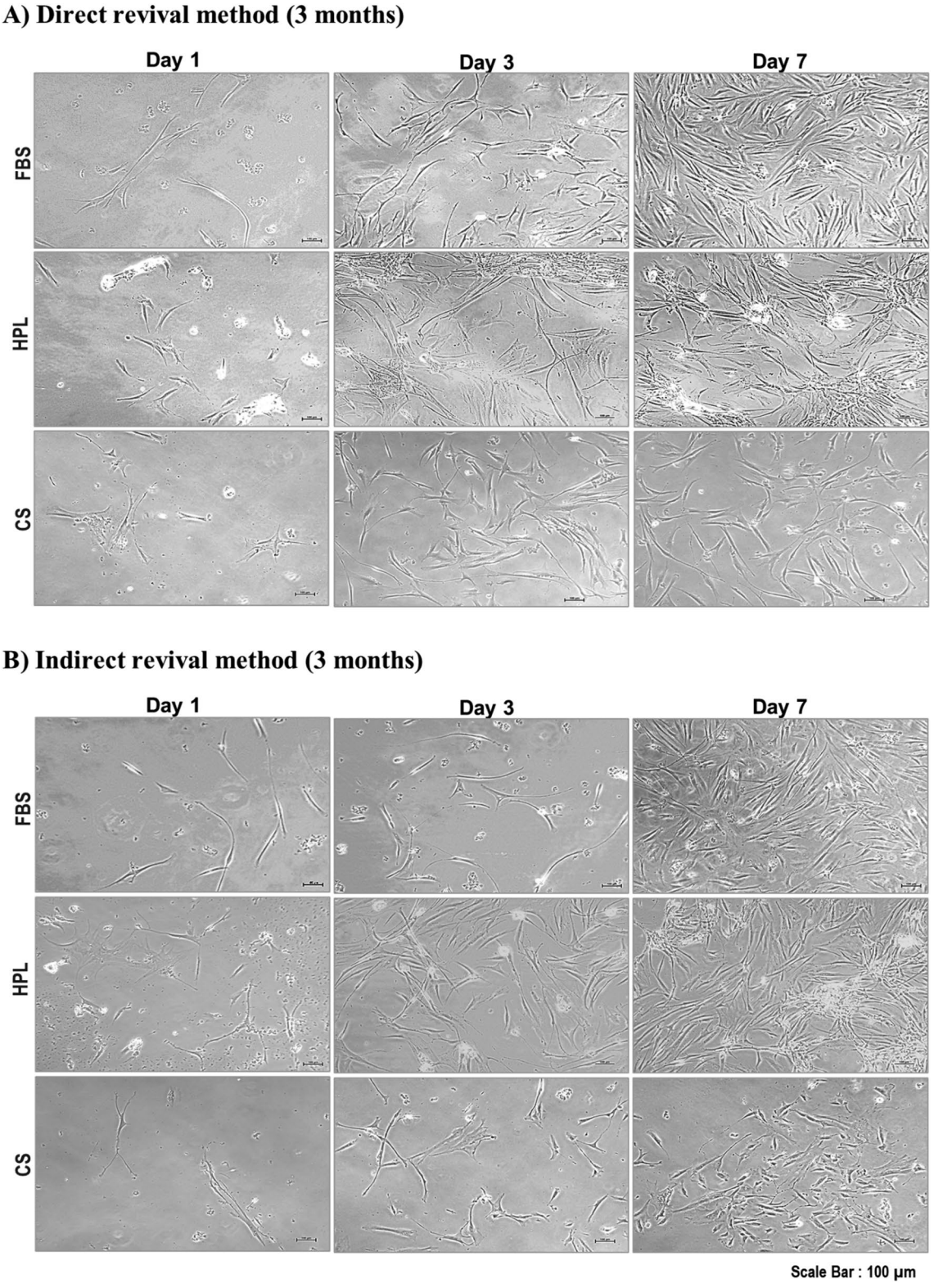

Fig. 4 Morphology of HDF after 3 months of cryopreservation using (**A**) direct and (**B**) indirect revival methods. The scale bar is 100 µm

observation suggests that these cells retained their morphology effectively even after 3 months of cryopreservation. Cells in the HPL cryo medium show an atypical clumping appearance in the media but still maintain the fibroblastic morphology. Fibroblasts in the FBS+10% DMSO group showed better cell attachment on day 1 in both revival methods compared to other groups and actively proliferated.

Immunocytochemistry (ICC) analysis

Immunocytochemistry (ICC) analysis was performed to evaluate the expression of specific protein markers in fibroblasts, examining any changes after cryopreservation. The results showed positive expression of Ki67 and Col-1 for both 1-month (Figs. 5) and 3-month (Fig. 6) cryopreservation, regardless of the cryo mediums and revival methods used. This indicates that the cells retained their specific protein expression after cryopreservation. Quantitatively, the percentage of positive expression for Ki67 and Col-1 was assessed across different cryo mediums and revival methods. For 1-month cryopreservation, there was no significant difference in Ki67 expression among the three cryo mediums for both revival methods. However, the percentage of Col-1 expression was significantly higher in cells preserved with FBS+10% DMSO compared to HPL+10% DMSO and Cryostor, regardless of the revival method. In contrast, for 3-month cryopreservation, the percentage of positive expression for both Ki67 and Col-1 was significantly higher in cells preserved with FBS+10% DMSO using the indirect revival method compared to other groups. This suggests that FBS+10% DMSO is the most suitable cryo medium for preserving fibroblast cells for 1 to 3 months while maintaining their natural phenotype.

Discussion

Cells or tissues product can be preserved for extended periods through cryopreservation [23]. Successful cell cryopreservation relies on proper freezing, sufficient storage, and accurate thawing methods [24]. Cryopreservation can lead to various types of cell damage through different mechanisms. Ice crystal formation, growth, and recrystallization are major limitations in cryopreserving cells, tissues, and organs, leading to severe damage to the biological samples [25]. Osmotic shock occurs as ice formation draws water out of cells, leading to cellular dehydration, shrinkage, and membrane damage [26]. Oxidative stress during cryopreservation also further damages cellular components. After thawing, cells must recover by controlling thawing to prevent ice recrystallization, gradually rehydrating to avoid osmotic shock, and activating repair mechanisms like DNA repair and antioxidant defenses [14]. Effective cryopreservation involves balancing these factors to minimize damage and aid recovery. Cryoprotectants like DMSO are commonly used to prevent ice formation, but high concentrations can be toxic, causing protein denaturation and metabolic disruption. According to other studies, the optimal DMSO concentration for cells, such as immortalized cell lines and hematopoietic stem cells, is typically between 5 and 10% [3, 27]. The usual cryo medium used in cell cryopreservation is a combination of either FBS, HPL or medium with the DMSO [28, 29].

In this study, the cell performances and viability in different cryopreservation conditions like different cryo mediums, storage durations, storage locations and cell revival methods were evaluated. Cell viability is defined as the existence of structural, metabolic and, for proliferating cells, reproductive and integrities essential for the preservation of life [24]. There are various methods to assess cell viability ranging from quantitative and qualitative measures, such as cell attachment, rate of cell growth, enzymatic activity and dye exclusion. Previous study showed that human primary skin cells can be stored via cryopreservation for up to 12 months and still retain their characteristics [1]. Fibroblast shows the highest number of vials with optimal cell attachment of more than 60% compared to other cell types as shown in Fig. 1A. Different cells respond and adapt differently to cryopreservation depending on their physical and biological properties, their origin donor as well as the metabolic condition and cell passages in an expanded primary cell culture [30]. Fibroblasts, known for their versatility and functional nature, have high viability due to their robustness, shorter doubling time, and ability to survive in vitro culture [31, 32]. On contrast, keratinocytes which are fully differentiated cells from the epidermis, grow well when cultured with fibroblasts with the synergistic effects of growth factors [33, 34]. The growth of keratinocytes benefits from being cultured with fibroblasts without using animal components and serum free medium [35, 36].

The attachment of revived cells that cryopreserved in FBS+10% DMSO showed a higher attachment rate, optimal total number of live cells, and better viability compared to those cryopreserved in commercial medium (Figs. 1B and 2). The morphological images of fibroblasts in Figs. 3 and 4 also show that fibroblasts in the FBS+10% DMSO group exhibited better cell attachment on day 1 in both revival methods compared to other groups. FBS provides essential nutrients, hormones, growth factors, amino acids, proteins, and other factors necessary for cell metabolism and proliferation, helping maintain cell viability during cryopreservation [37]. This is crucial for cells to survive the stress of freezing and thawing. Previous findings by Fugisawa et al. (2019) also reported that FBS helps balance osmotic pressure during freezing and reduces ice crystal damage [38]. FBS is one

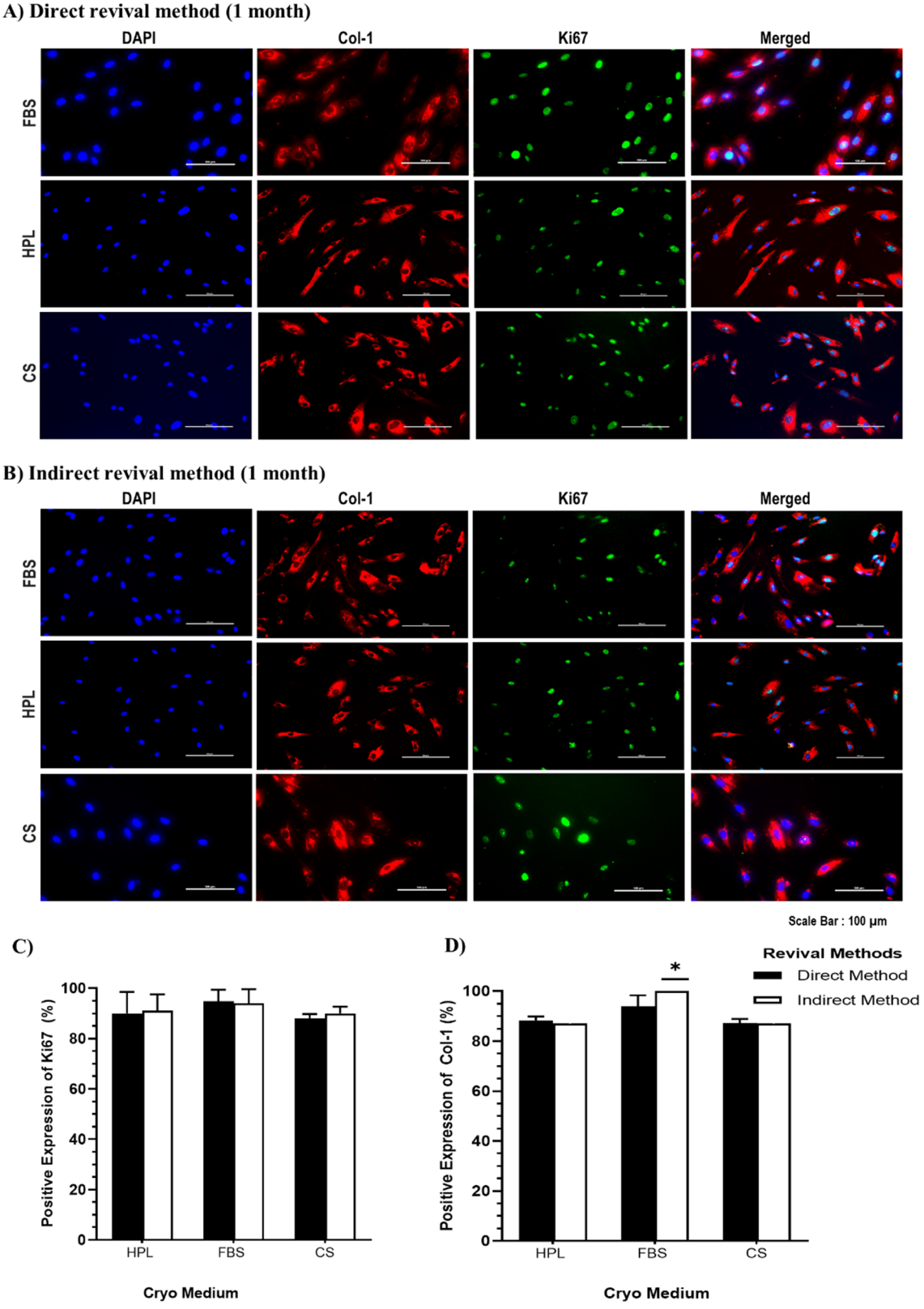

Fig. 5 Immunocytochemistry (ICC) images of HDF after 1-month of cryopreservation with different cryo mediums and revival methods: (**A**) direct revival and (**B**) indirect revival. The proliferative markers Ki67 (green) and Col-1 (red) were strongly expressed. The scale bar represents 100 µm. At the bottom is the graph showing the percentage of positive expression in ICC for (**C**) Ki67 staining and (**D**) Col-1 staining. * Indicates a significant difference compared to HPL + 10% DMSO and Cryostor with both revival methods, with $p < 0.05$

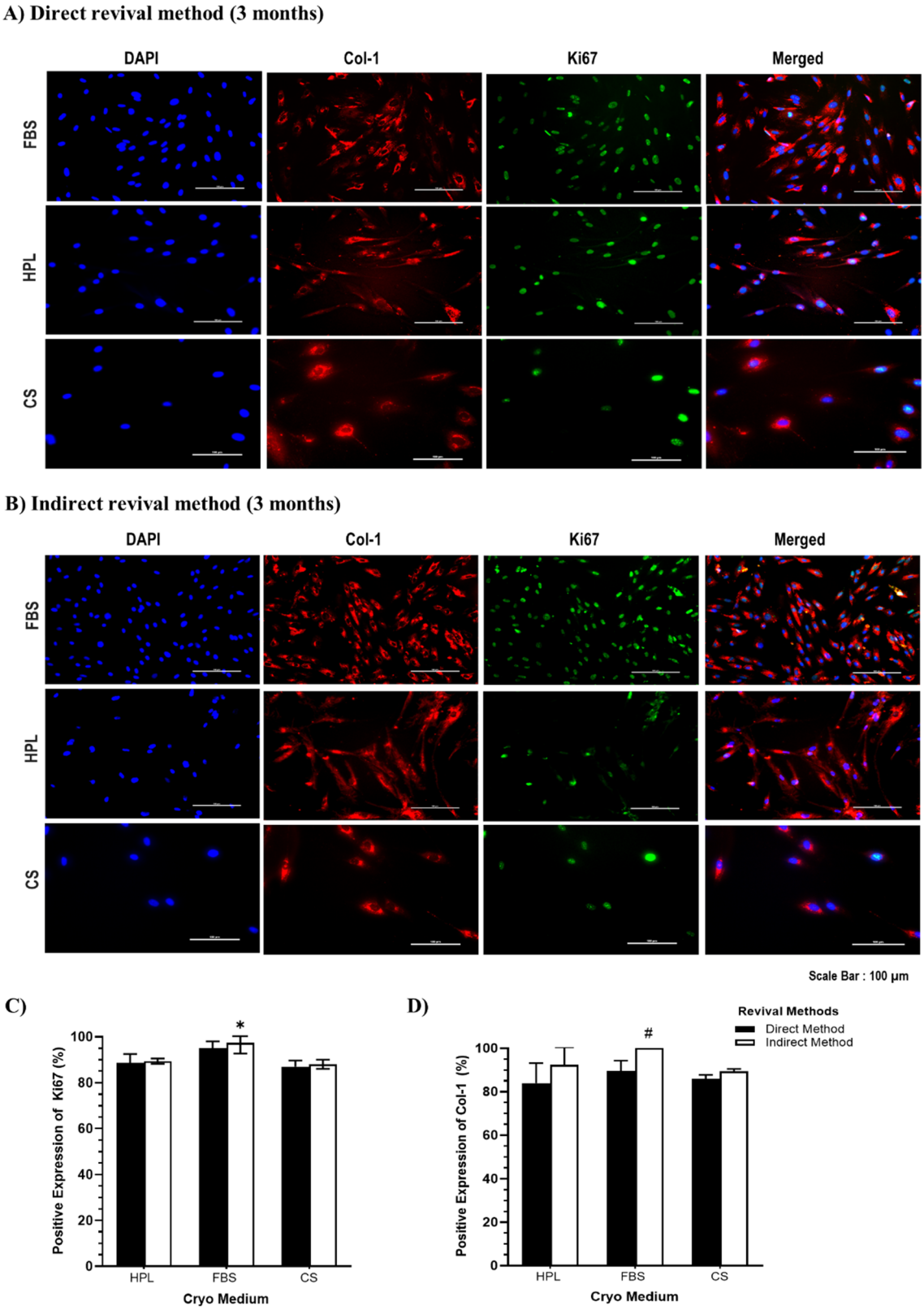

Fig. 6 Immunocytochemistry (ICC) images of HDF after 3-months of cryopreservation with different cryo mediums and revival methods: (**A**) direct revival and (**B**) indirect revival. The proliferative markers Ki67 (green) and Col-1 (red) were strongly expressed. The scale bar represents 100 µm. At the bottom is the graph showing the percentage of positive expression in ICC on (**C**) Ki67 staining and (**D**) Col-1 staining. ***** Indicates a significant difference compared to HPL + 10% DMSO and Cryostor with both revival methods. # Indicates a significant difference compared to HPL + 10% DMSO with the direct method. $p < 0.05$

of the earliest and most commonly used agents in cryopreservation, especially in fields involving mesenchymal cells [39]. According to Duarte Rojas JM et al. (2024), cryopreservation and thawing of cells is more effective in either platelet lysate serum or FBS [37]. Nonetheless, the advancement in cryopreservation have exposed established risks associated with the use of calf-harvested serum such as xenogenic materials, transmission of animal to human infectious disease and immunizing effect that compromise the cells' quality and biosafety in clinical application and therapeutic outcome [36, 40].

An alternative of serum-free media or synthetic mediums, and human-derived serums, plasma or platelet derivatives become safer preferences with impressive cell viability, sterility and extensive expansion with stable immune phenotype, differentiative and immunomodulatory, which is ideal for clinical applications [40]. The synthetic mediums are chemically defined, offering consistency, and can be tailored to different cell types improving their survival and functionality [41]. In this study, cells cryopreserved in a commercial cryo medium showed lower viability might be due to the defective CoolCell freezing container (Mr. Cool) used, which couldn't close properly, leading to suboptimal cooling and stress to the cells. Additionally, a study reported that the percentage of cell recovery slightly dropped after being stored in commercially available medium for 5 months of cryostorage compared to 1 month, with a slight increase in caspase 3 activity, an indicator of apoptotic cells [42]. Therefore, the suitability of the cryomedia, as well as the standardization of the storage and handling processes, is crucial to ensure consistent cooling rates and minimise thermal stress, thereby preserving cell viability and functionality post-thaw.

Prolonged cryopreservation duration can affect the long-term viability [1] and genetic stability of cells. It is known that low temperatures stop the metabolic activity of cells, and cryoprotective agents are used to protect cells from damage caused by freezing temperatures. However, the longer the storage duration, the more cells are exposed to temperature fluctuations, the effectiveness of the cryo medium, and potential issues from the storage conditions themselves [7]. In this study, majority of the cells able to reach more than 60% cell attachment in 24 h for the 0–6 months storage durations whilst cells stored for more than 24 months have the most numbers of vials with less than 20% cell attachment (Fig. 1C). For short-term cryopreservation studies, the viability of HDF post-cryopreservation at 1 and 3 months showed a slight decrease trend compared to before cryopreservation, but the cells still actively proliferated within 7 days of culture (Fig. 2). This might be due to disruptions in the ultra-low temperature environment, such as issues with cryoprotective agents or external factors like frequent opening of nitrogen tanks, can cause cryoinjury to cells. This injury leads to osmotic imbalances, crystal formation, and damage to cell membranes and organelles, which are crucial for cell viability [7].

The method used to revive cells after cryopreservation also could affect cells viability. The main difference between the direct and indirect methods is the concentration of the cells obtained. The indirect method uses centrifugation to concentrate cells pellet, increasing viable cell yielded, removing the DMSO and reducing the contamination. However, the direct method has the more vials with more than 60% cell attachment compared to the indirect method mostly in 0–20% cell attachment (Fig. 1D). The direct method used is more effective in optimising the viability of cells after cryopreservation because centrifugation step can cause mechanical stress and disrupt osmotic balance, leading to cell clumping and loss [43]. However, the HDF stored for 1 and 3 months showed no significant differences in terms of the total number of live cells and viability after being revived using both methods (Fig. 2). The cells also retained their elongated and spindle-shaped morphology, indicating that their characteristics and ability to proliferate were maintained after thawing (Figs. 3 and 4). This might be due to the robustness of the fibroblasts and the short storage duration, along with the centrifugal steps.

The storage location can also affect the effectiveness of cryopreservation by exposing cells to different chemical states of liquid nitrogen. Historically, cells fully immersed in liquid nitrogen are more viable because the liquid state is more stable, resulting in less temperature fluctuation compared to vapor or mixed state. This stability reduces the risk of cryo injury, such as cellular dehydration, intracellular crystal formation, and cell death caused by supercooling [44]. Figure 1E and F show that more vials stored in boxes 2 and 3, which are in the vapor phase, have more than 60% cell attachment. With the advent of better-designed storage vessels, storing in the liquid phase has become unnecessary. The vapor phase is preferred because it avoids the risks associated with liquid-phase storage, such as containers potentially exploding when removed, cross-contamination by viruses in the liquid, and exposure of operators to the extremely cold liquid [45]. However, this data does not confirm that vials immersed in liquid nitrogen have the poorest performance. When evaluating frozen storage containers, factors to consider includes the application type (research or clinical), fill volume, temperature, aseptic filling, access for removal, sterility, biocompatibility, potential regulatory requirements, and scalability [30].

Optimising multiple factors during cryopreservation and thawing increases the likelihood of successful cell recovery post-thawing [46]. The evaluation of specific protein expression is essential to characterise and

identify if there are any changes in the cryopreserved cells. ICC analysis was performed to evaluate the protein expression of Col-1 and Ki67. Collagen type I is a common protein in the body, making up a large part of bones, ligaments, tendons, and skin which is specific marker that mainly produced by fibroblasts [47–49]. Ki-67 helps organise cellular structures during interphase and protects chromosomes during cell division, preventing clumping [50, 51]. By measuring Ki-67 expression, the ability of fibroblast cells to proliferate and remain viable after being frozen and thawed was assessed. The cells without positive Ki67 expression maybe stuck in the G0 or G1 phase of the cell cycle during stabilisation [52, 53]. After 1 and 3 months of cryopreservation, both markers were expressed by HDF (Figs. 5 and 6) in all types of cryo medium with more than 80% for both revival methods which confirm HDF ability to retain its characteristics [1, 54–56].

In this study, obtaining data on the type of cryo medium used based on previously collected data posed its own set of challenges, including incomplete records, inconsistencies in the documentation of cryopreservation conditions, and variability in the formulations of cryo medium. Besides, the defective container (Mr cool) resulted in suboptimal cooling rates, and thermal cycling from repeated exposure to varying environmental conditions further stressed the cells. This combination of factors led to reduced cell viability after thawing. Inconsistent handling and storage procedures, transient exposures to non-ideal conditions, and inadequate monitoring of temperature and environmental factors all contributed to compromised cell integrity. Thus, with the advancement of alternative cryoprotective mediums and the meticulous optimisation of storage techniques, cryopreserved cells can maintain their integrity and safety for research and therapeutic use.

Conclusion

Optimising cryopreservation conditions is crucial for keeping cells viable, functional, and genetically stable. This study successfully determined the best cryopreservation conditions for human fibroblasts and other human cells to achieve optimal viability and growth. The cells maintained their shape and specific protein expression. Despite a decrease in live cell numbers and viability for both 1 and 3-month cryopreservation periods, the cells retained over 55% viability and more than 400,000 cells per vial, regardless of the cryo medium or revival method used and able to proliferate within 7 days of culture. Cryopreservation has significant potential for basic research and medical applications, including tissue engineering and regenerative medicine. It can address challenges in cell banking, such as maintaining cell integrity and functionality across different storage conditions. Further research is needed to explore longer cryopreservation periods (e.g., 12 or 24 months) and other cryo mediums, and to evaluate additional cell types and characteristics.

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Author contributions
Conceptualization, N.S, and M.M.; validation, H.M.M., P.S., N.A.A.R., A.J.C., N.A.M.S, N.S, and M.M.; writing—original draft preparation, H.M.M., P.S., N.A.A.R., A.J.C., N.A.M.S, and M.M; drawings of figures, H.M.M., P.S., N.A.A.R., A.J.C.,; writing—review and editing, H.M.M., P.S., N.A.A.R., A.J.C., N.A.M.S, N.S, M.B.F., and M.M.; visualization and supervision, N.S., M.B.F., and M.M.; project administration, M.M.; funding acquisition, M.M. All authors have read and agreed to the latest version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study is conducted following UKM research ethics committee approval with the reference number: UKM PPI/111/8/JEP-2023-007. The cryopreserved cell for this study was obtained from DTERM primary cell bank with ethics approval number: [UKM1.5.3.5/244/FF-2015-376] and patient consent.

Consent for publication
The author read and approved the final manuscript for publication.

Informed consent
The authors declare that informed consent was taken based on the approval of UKM research ethics approval number: [UKM1.5.3.5/244/FF-2015-376].

Competing interests
The authors declare no competing interests.

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