Sains Malaysiana 52(11)(2023): 3103-3119
http://doi.org/10.17576/jsm-2023-5211-07

The Optimal Cryo Revival Period of Cryopreserved Wharton's Jelly Derived-Mesenchymal Stem Cells

(Tempoh Pemulihan Krio Optimum bagi Sel Stem Mesenkima Wharton Jeli yang Diawet Krio)

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Received: 12 May 2023/Accepted: 20 October 2023

ABSTRACT

The workflow of cryopreservation is a challenging step in the standardised preparation of cell therapy products in terms of methods used (i.e., adapted controls of the work environment, quality control, reagents, and equipment). This study aimed to determine the effect of cryopreservation on the stability of mesenchymal stem cell (MSC) characteristics by comparing fresh cells with those that underwent different post-thaw cell recovery periods. The MSCs were derived from human Wharton's jelly umbilical cord (n = 4). The cells that were cryopreserved for 7 days were revived at 0 h (CRC-0h), 24 h (CRC-24h) and 7 days (CRC-7d) and then evaluated on the basis of cell viability, doubling time, morphology, trilineage differentiation potential, growth kinetics, and MSC surface marker expression. The cell viability of the CRC-0h group was 90%, whereas that of the CRC-24h group was 80%-85%. Cell attachment results showed that CRC-24h had a notably higher attachment rate than the other two groups. The CRC groups showed CD90, CD73 or CD44 expression, which meets the minimum criteria for defining multipotent MSCs. By contrast, CD105 expression was significantly reduced in the CRC groups and was lower than the minimum requirement based on the standards of the International Society for Cellular Therapy. Results suggest that at least 24 h is necessary to improve the quality of MSCs for it to be adequate for cell therapy use.

Keywords: Cell therapy; cryopreservation; cryorevival; mesenchymal stromal cell; recovery period

ABSTRACT

Aliran kerja pengawetan krio ialah satu langkah yang mencabar dalam penyediaan piawai produk terapi sel daripada segi kaedah yang digunakan (seperti kawalan disesuaikan persekitaran kerja, kawalan kualiti, reagen dan peralatan). Kajian ini bertujuan untuk menentukan kesan pengawetan krio ke atas kestabilan ciri sel stem mesenkima (MSC) dengan membandingkan sel segar dengan sel yang menjalani tempoh pemulihan sel pasca nyahbeku yang berbeza. MSC diperoleh daripada tali pusat jeli Wharton manusia (n = 4). Sel yang diawet krio selama 7 hari telah dihidupkan semula pada 0 jam (CRC-0j), 24 jam (CRC-24j) dan 7 hari (CRC-7h) dan kemudian dinilai berdasarkan daya maju sel, masa gandaan, morfologi, potensi pembezaan *trilineage*, kinetik pertumbuhan dan ekspresi penanda permukaan MSC. Daya maju sel kumpulan CRC-0j ialah 90%, manakala kumpulan CRC-24j ialah 80%–85%. Keputusan pelekatan sel menunjukkan bahawa CRC-24j mempunyai kadar pelekatan yang lebih tinggi daripada dua kumpulan lain. Kumpulan CRC menunjukkan ekspresi CD90, CD73 atau CD44 yang memenuhi kriteria minimum untuk menentukan MSC multipoten. Sebaliknya, ekspresi CD105 berkurang dengan ketara dalam kumpulan CRC dan lebih rendah daripada keperluan minimum berdasarkan piawaian *International Society for Cellular Therapy*. Keputusan menunjukkan bahawa sekurang-kurangnya 24 jam diperlukan untuk meningkatkan kualiti MSC supaya ia mencukupi untuk kegunaan terapi sel.

Kata kunci: Pemulihan krio; pengawetan krio; sel stromal mesenkima; tempoh pemulihan; terapi sel

Introduction

Stem cells, which are unspecialised cells with the capacity for self-renewal and the ability to split into specialised or multiple cell types, comprise every tissue in the human body. Stem cell therapy or regenerative medicine uses the regrowth of organs or tissues to treat or repair a wide range of degenerative illnesses in humans and animals. However, it has its limits and raises some ethical questions. This procedure is accomplished by using stem cells to activate the repair response of malfunctioning or damaged tissue (Biehl & Russell 2009; Ishak et al. 2019).

In the past year, mesenchymal stem cells (MSCs) have been used in various regenerative medicine applications. The US National Institutes of Health (http://www.clinicaltrial.gov/) reported that as of June 2015, 493 MSC-based clinical studies had either been completed or could exhibit the promising immunomodulatory capabilities and immunosuppressive properties of MSCs, thus making them a good choice for allogeneic regenerative medicine (De Miguel et al. 2012; Squillaro, Peluso & Galderisi 2016). Saeedi, Halabian and Imani Fooladi (2019) highlighted the unique characteristics of MSCs, including their ease of isolation and cultivation, their ability to differentiate into specific cell types and their function as bioreactors for soluble factors that can induce migration (homing) to injured tissues. Bone marrow (BM) MSCs and umbilical cord (UC) MSCs are the two most common MSCs studied previously (Chen et al. 2021; Selich et al. 2019).

McElreavey et al. (1991) were the first to identify human Wharton's jelly (hWJ) as a rich source of MSCs. Moreover, hWJ has proven to be a rich source of MSCs when compared with other sources (Kassem & Kamal 2020). UC sources have become advantageous for potential therapeutic applications due to the ease of extracting MSCs from hWJ-MSC tissues and the significant *ex vivo* growth capacity achieved in a single culture. Kern et al. (2006) found that isolating MSCs sometimes led to experimental group failures, making this source donor dependent, with each batch exhibiting sample variation.

Over the last 20 years, the number of clinical uses for MSCs has grown, resulting in a more in-depth study of MSCs during the recovery period; moreover, the development of ways to improve cell recovery for immediate or ready-to-use hWJ-MSC-based products has become an absolute necessity in regenerative medicine. This move is essential to preserve the functional capacity of the stem cells for long-term storage until therapy with a conditioning regimen (Shivakumar et al. 2015). Cryopreservation is categorised into slow freezing, also known as the conventional cryopreservation technique, and vitrification, which has recently been recognised as a suitable method for tissue cryopreservation. Some researchers have reported that slow freezing also has the potential for tissue cryopreservation. Although conventional cryoprotective agents (CPAs) have been well studied, some novel ones may efficiently cryopreserve tissues via slow freezing. Shivakumar et al. (2015) reported that the vitrification (rapid cooling) of hUC-MSCs is a reliable and effective method of cryopreservation but consumes a high concentration of CPAs. In this study, optimal recovery period after cryopreservation of hWJ-MSCs using the conventional slow freezing technique was investigated.

Conventional cooling rate methods using CPAs introduce several distinct issues. These problems are related to CPAs and include freezing, freezing temperature, thawing timing and the gradual removal of CPA concentration during the post-thaw recovery period (Gurruchaga et al. 2018). These factors should be carefully considered when working with hWJ-MSCs to ensure their stability during the cryorevival process. Whilst freshly harvested hWJ-MSCs can undergo freeze–thaw cycles, this process may still affect cell membrane physiology. The effectiveness of cryopreservation depends on various factors, and even with successful cryorevival, some inevitable loss in cell viability may occur post thaw. Standardising the optimal revival period can enhance the quality of hWJ-MSC cryorevival before clinical use. To assess the quality of hWJ-MSC production, the viability of post-thaw cells with fresh cells (FCs) must be compared using conventional cryopreservation procedures.

In the context of a stem cell bank with a long-term preservation goal spanning decades, ensuring optimal clinical utility and consistent research outcomes necessitates a robust quality control system. Thus, this study focuses on the importance of maintaining such system for stem cell banks with long-term preservation goals. Specifically, the research aims to assess the effect of cryopreservation on hWJ-MSCs, which are known for their self-renewal abilities and high proliferative potential, making them valuable for maintaining stemness even after the revival and culture processes. The study aligns with established protocols for isolating, characterising and expanding MSCs, all of which meet the minimum criteria set forth by the International Society for Cellular Therapy (ISCT) (Kannaiyan et al. 2017).

The primary objective of the study was to identify the optimal recovery period for hWJ-MSCs, emphasising their stability during the cryorevival process. The study seeks to determine how the stability of MSC characteristics is affected by cryopreservation. This goal is achieved by comparing FCs with cells recovered at different post-thaw assessment periods, specifically at 0 h (CRC-0h), 24 h (CRC-24h) and 7 days (CRC-7d) after cryopreservation. This assessment includes a comprehensive examination of cellular morphology, cell viability, total living cell counts, MSC-specific marker expression, multilineage differentiation potential and growth curve analysis of cryorevived cells (CRCs). The goal is to enhance our understanding of how cryopreservation affects hWJ-MSCs and contribute to the development of improved preservation and utilisation strategies for these valuable stem cells.

Materials and Methods

Dulbecco's modified Eagle medium (DMEM)-low glucose (DMEM-LG), minimum essential alpha medium, Dulbecco's phosphate buffered saline (DPBS) and antibiotics–antimycotics were obtained from Gibco, USA. Foetal bovine serum (FBS) and TrypLE™ Select Enzyme (1X) without phenol red were obtained from Gibco, New Zealand. Trypan blue solution; 4% paraformalxrdehyde; 50%, 95% and 100% alcohol; acid alcohol, ammonium and 70% alcohol were used. During cryopreservation, Cell Freezing Medium (ScienCell, USA) was used to freeze the cells. Before the digestion of tissue fragments, they were treated with 10% povidone; Sigma 0.6% collagenase type-1 powder was used for enzymatic digestion. Hoechst stock solution (2 µM) was used to stain the living cells. A stain buffer (shield fluid) and a BD human MSC analysis kit (positive cocktail, negative cocktail, isotype) were used for immunophenotype analysis. For MSC trilineage differentiation, the following materials were used: StemPro osteogenic differentiation basal medium, StemPro osteogenic supplement, StemPro chondrogenic differentiation basal medium, StemPro chondrogenic supplement, StemPro adipocyte differentiation basal medium and StemPro adipocyte supplement. For staining, the following materials were used: 2% alizarin red S solution (Sigma–Aldrich), solution (Science-cell), 0.5 g oil red O powder (Sigma–Aldrich), 50% ethanol, isopropanol alcohol (IPA), distilled water, 60% IPA and sterile filter Mili-Q water.

Equipment and Tools

Sterilised surgical tools included forceps, scalpel and a kidney dish. A magnetic stirrer bar, a pH metre, a sterile filtration unit and 0.22 µm porous nylon membrane filter were used to prepare the culture medium. The following basic equipment were available for use: Class II BSC (BioAirSafemate, Italy), CO^2 incubator (37 °C with humidified atmosphere of 5% CO^2), New Brunswick 37 Galaxy® 170 Series (Eppendorf, USA), centrifuge and micropipettes (Eppendorf, Germany), pharmaceutical refrigerator with freezer and −20 °C freezer (Panasonic Sanyo, Japan), shaker incubator and heating block (Stuart, UK), 37 °C water bath (Benchmark Scientific, USA), inverted and light microscope (Olympus, Japan), pipette gun, weight scale machine, vortex and autoclave. To count cells, a haemocytometer that was obtained from Neubauer Laboroptik, UK was used. Tubes (15 and 50 mL), serological pipettes (5, 10 and 25 mL) and culture container T-flasks (T-25 cm^2, T-75 cm^2, T-175 cm^2) were obtained from Corning, USA. Six-well and 12-well culture plates were obtained from Thermo Scientific, Denmark. Pipettes (p1000, p200, p10) were obtained from Eppendorf. For cryopreservation, Cryo 2 mL vials and Mr. Frosty Nalgene® BD Falcon round-bottom 12 × 75 mM polystyrene tubes with caps were used. A BD FACSVerse™ flow cytometer machine was obtained from Backton Dickinson, USA. A Nikon Eclipse Ti inverted fluorescence microscope (Nikon, Japan) was also used.

Ethics Approval and Sample Collection

This study used four human umbilical cord samples. The Faculty of Health Sciences, Universiti Selangor (UNISEL) (J160364E) and the Universiti Kebangsaan Malaysia (UKM) Research Ethics Committee (UKM PPI/111/8/JEP-2020-506) approved the study. The samples were collected from full-term pregnant women undergoing labour at the Department of Gynaecology and Obstetrics at Hospital Canselor Tuanku Muhriz, UKM.

Preparation of Primary hWJ-MSCs

The primary cells were isolated from the human umbilical cord, specifically from Wharton's jelly, using the collagenase type-I method, in accordance with a previous paper (Zhao et al. 2015). On the third day of culture, primary cells exhibited migration, forming a monolayer of cells. These primary cell lines of MSCs were cultured until they reached approximately 70%–80%

confluency in the culture flasks, spanning from Passage 1 to Passage 3 (P1–P3). The primary cell lines of hWJ-MSCs were harvested at Passage 3 once sufficient confluency was achieved. At Passage 3, hWJ-MSCs were pooled, and experiments were conducted in triplicate (n = 3).

SPECIMEN PROCESSING AND ISOLATION OF hWJ-MSCs

Culture media were changed every three days. When the cell density reached 80%, the cells were removed from the surface of the flask using trypsin–EDTA solution (0.05%), which was then expanded into a new flask with a density of 5×10^3 cells/cm^2. To ensure uniformity of cell use, cells in subculture three (P3) were used throughout the experiment. Although the MSCs isolated from the Wharton's jelly portion of the human umbilical cord in this study were unpurified MSCs, a previous study at the Center for Tissue Engineering, UKM had characterised the isolated cells using the method described above (Lim et al. 2021).

hWJ-MSC CULTURING

DMEM was replaced after the cell attached approximately 2 to 3 days. The cells' confluency was observed using the inverted microscope, and a visual check on the cells was performed. When the cell density reached 80%, cells were washed twice with DPBS, and trypsin solution was added into each tissue culture flask, and the cells were allowed to detach for 5 min at 37 °C. Cells were transferred into a 15 mL centrifuge tube, where they were centrifuged at 5000 rpm for 5 min to obtain a cell pellet. The supernatant was discarded, and a cell pellet suspension was prepared to determine the cell viability. Then, the hWJ-MSCs was seeded at density 5×10^3 cells/cm^2 in a T-75 flask (Yi et al. 2020). To ensure uniformity of cell used, cells in subculture three (P3) were used throughout the experiment. The MSCs isolated from the Wharton's jelly portion of the human umbilical cord in this study were unpurified MSCs.

PREPARATION OF hWJ-MSCs FOR POST-THAW ASSESSMENT

To prepare hWJ-MSCs for post-thaw assessment, cells at P3 were obtained, resulting in a 10×10^6 cell suspension for each group. These groups consisted of FCs, known as noncryopreserved cells, and CRCs, which underwent post thawing. The FC group underwent continuous culture, whereas the CRC groups were categorised as follows: CRC-0h, CRC-24h and CRC-7d. Cells were cryopreserved by placing cryovials in precooled isopropanol racks (Mr. Frosty, Nalgene®) and transferring them to a −80 °C freezer for 24 h prior to final liquid nitrogen phase (−196 °C). After 1 week of storage, the cells were thawed for further processing or testing. On the day of experimentation, the cells were immediately thawed, creating CRC-0h cells, and sample analysis proceeded. Afterwards, CRC-24h and CRC-7d cells were thawed. Cell viability assay was conducted, and the 10×10^6 cells were transferred into T-75 flasks (n = 5). CRC-24h cells were harvested after one day, whereas CRC-7d cells were harvested after one week. Cryopreservation was performed for all hWJ-MSC groups, except for the FC group. After a one-week period, the vial of hWJ-MSCs was removed from liquid nitrogen and incubated in a 37 °C water bath. Thawed hWJ-MSCs were then prepared for post-thaw assessment, which included morphology studies, total cell count and cell viability comparisons between the CRC and FC groups. Additionally, the CRC and FC groups were evaluated in terms of MSC marker expression, multilineage differentiation potential and growth curve analysis.

MORPHOLOGICAL OBSERVATION

Photographs of the cultures were captured using an inverted CK40 microscope (Olympus, Germany) equipped with an integrated camera system. The microscope was set to the appropriate magnification, and images of fresh and post-thaw hWJ-MSCs were taken for comparative analysis. The camera system allowed for precise image capture and documentation of cell morphology.

CELL VIABILITY AND TOTAL NUMBER OF CELLS

After trypsinisation, the cell suspension was homogenised and mixed with trypan blue; a haemocytometer was used to count the number of cells. On the haemocytometer at 40× magnification, all cells were counted inside each of the four sizeable quadrants in the four corners of the counting chamber. To calculate the percentage of the vitality of MSCs, the number of living cells was divided by the sum of all cells (Shivakumar et al. 2015).

MSC PHENOTYPING AND MULTILINEAGE DIFFERENTIATION ASSAY

The cells were suspended in the medium at a concentration of 1×10^6 cells/mL and were then stained with a panel of antibodies from the BD Stemflow™ human MSC analysis kit (San Diego, CA; PMG562245).

This panel included a negative MSC cocktail (PE CD45, PE CD34, PE CD11b, PE CD19 and PE HLA-DR) and a positive cocktail (FITC CD90, PerCP-CyYM5.5 CD105 and APC CD73). Multilineage differentiation assay was conducted using commercially available kit from Gibco, USA, which included the StemPro chondrogenesis differentiation kit (Catalogue Number: A1007101), StemPro osteogenesis differentiation kit (Catalogue Number: A1007201) and StemPro adipogenesis differentiation kit (Catalogue Number: A1007001).

GROWTH CURVE OF CRC AND FC

Cell adhesion and proliferation were examined using a 1:1 ratio of Hoechst dye. For this analysis, hWJ-MSCs at P3 were cultivated in triplicate in 12-well plates, with a cell density of 2×10^3 cells/cm^2, over the course of 1, 3, 5, 7 and 9 days. Cell attachment and proliferation were assessed using a fluorescent microscope, and image analysis was performed using an algorithm detection method (Nikon A1R, Japan).

STATISTICAL ANALYSIS

Data were extracted from the hands-on laboratory result: control group, outcome of experiment groups, method of outcome evaluation, main finding and conclusion. Descriptive summaries of studies included were entered into tables. Statistical analysis and evaluation of the results concerning hWJ-MSC post-thawed in different time frames (CRC-0h, CRC-24h, CRC-7days) were performed, and results were compared using one-way ANOVA. $p < 0.05$ was considered statistically significant. MSC marker expression between CRC and FC groups was compared using two-way ANOVA. Data from each experimental group were analysed using GraphPad Prism version 7.0 (GraphPad Software, Inc., USA).

RESULTS

MORPHOLOGY AND VIABILITY OF THE PRIMARY hWJ-MSCs

Primary cells were obtained from hWJ (n = 4) and seeded in DMEM-LG (Gibco, USA) using high-density cell culture, resulting in numerous fresh live cells with high expansion and differentiation potential. Many cells attached and migrated to the surface of flasks after 7–12 days of enzymatic culture and were grown in media supplemented with FBS. The culture plates showed the absence of contamination. Adherent cells could proliferate, and when they reached 70% confluence, they were subcultured. Phase contrast microscopy showed cells resembling a combination of thin and long bipolar or fibroblastic cells (Figure 1(A)). P0 cell division began from tissue fragments (red arrow) and flattened enlarged cells in P0 early passage (black arrow). P1 showed a mixture of cell development, including long flattened cells (red arrow) and tripolar-shaped cells (black arrow). More cells expanded into tripolar-spindle-shaped cells at P2 (black arrow). Cells at P0 until P3 showed that the hWJ-MSCs maintained fibroblast-like morphology in the presence of a few large tripolar-shaped cells. The viability of hWJ-MSCs was maintained at 70%–90%, 73–88%, 80–90% and 85–92% at >70% at, P0, P1, P2 and P3, respectively (Figure 1(B)).

POST-THAW ASSESSMENT OF CRC hWJ-MSCs BETWEEN 0 H, 24 H AND 7 DAYS

To investigate the effects of cryopreservation at 0 h, 24 h, and 7 days, hWJ-MSCs were thawed and cultured for cell morphology studies, cell viability, total cell count, and MSC immunophenotype expression. The CRC group was maintained at P4 to limit variation in cell populations. A total of 10×10^6 cells were plated in each 175 cm^2 flasks. Under a microscope, CRC-0h cells appeared rounded and were suspended in the media. After 24 h, cells were observed to be attached at 20× magnification (Figure 2(B), 2(E)). The CRC-24h culture contained irregularly shaped cells and debris that either sedimented or floated in the medium, indicating dead cells that could not recover from cryopreservation. On day 7, cells had reached 70% confluence, and most of the cells were adherent with a thin spindle shape at P4 (Figure 2(C), 2(F)). CRC-7d hMJ-MSCs exhibited a similar spindle structure to freshly cultured hWJ-MSCs, but the confluence was comparatively lower than the FC group. Day 7 significantly improved the post-thaw recovery of viable hWJ-MSCs compared with the FC hWJ-MSCs.

The viability assay and total nucleated cell (TNC) count of CRC hWJ-MSCs showed a significant reduction in all the cryopreserved groups compared with the FC group, as determined by two-way ANOVA. Immediately after thawing, the CRC-0h group showed a significant reduction in hWJ-MSC viability compared to fresh hWJ-MSCs (FC) ($p > 0.0001$). The mean viability of these cryorevived samples was 96.25% (min 93.53%, max

98.97%), as observed in a pooled sample at P4 after undergoing freezing and thawing (Figure 3(A)). However, the cell viability of CRC-24h showed a statistically lower recovery compared with other groups. Furthermore, CRC-24h exhibited significant growth reduction after one day to $< 1.0 \times 10^7$ (p 0.0067) (Figure 4(B)).

By day 7, the hWJ-MSCs demonstrated increased proliferation compared with CRC-24h. The CRC-7d group showed a cell viability of over 80%, similar to the FC group, indicating remarkable metabolic activity in the CRC. Differences in viability were no longer significant at day 7; the cell culture showed higher viability than other CRC groups ($p > 0.0001$). Notably, the number of viable cells was not affected by cryopreservation and retained growth, as observed in the FC group after a seven-day culture.

MSCs MARKER EXPRESSION ON CRC hWJ-MSCs

The flow cytometry analysis of P4 hWJ-MSCs showed homogeneous reactivity, with negative results for CD34, CD45 and HLA-DR, and consistent positivity for MSC markers, such as CD44, CD90 and CD105. Notably, the CRC group exhibited slightly different results depending on the recovery period (Figure 4(A), 4(B)). The percentage of positive marker expression was similar and moderately stable between the CRC and FC groups.

Two-way ANOVA showed that CD90 (99.86%–97.69%), CD73 (99.88%–99.67%) and CD44 (84.06%–40.50%) consistently maintained positive MSC marker expression across all groups, except for CD105 (84.06%–40.50%), which did not meet the expected range of >95% according to ISCT guidelines. As shown in Figure 4(B), these negative antigens were not typically found on CRC hWJ-MSCs at 0 h (0.16%), 24 h (0.28%) and 7 days (0.50%); however, they remained below 5%. The results demonstrated moderate stability in all CRC group populations compared with surface markers. The percentage expression of these markers was similar between the control group and the CRC-7d group.

MULTILINEAGE DIFFERENTIATION POTENTIAL ON CRC hWJ-MSCs

The osteogenic differentiation potential of hWJ-MSCs, as assessed by calcium accumulation, was determined through Alizarin red-positive staining. Refractile substances were observed in the cell colonies, and small round nodules were detected in the FC and CRC groups (Figure 5(C), 5(F)). Similarly, chondrocyte cells were formed in the pellet for CRC and FC groups (Figure 5(I), 5(L)), indicating a positive result, which was further confirmed by safranin O staining. In the case of adipogenic differentiation, the hWJ-MSCs displayed the presence of small fat droplets in the cytoplasm after 21 days of induction in the FC and CRC groups (Figure 5(O), 5(R)).

In summary, the differentiation potential of the cells is a crucial criterion for characterising them as MSCs, encompassing adipogenic, chondrogenic and osteogenic differentiation abilities under specific culture conditions.

COMPARING THE GROWTH CURVE BETWEEN THE FC AND CRC GROUPS

After day 1, the CRC group (1,487 cells/cm^2) showed a slightly slower rate of cell attachment to the flask surface compared with the FC group (1,553 cells/cm^2). The FC group exhibited the highest total number of living cells attached on day 9 (10,425 cells/cm^2), followed by the CRC group (8,723 cells/cm^2). The doubling time of FC (153.44 h) and CRC (129.14 h) was higher on day 7, indicating the logarithmic growth phase. A distinct inhibitory trend in cell proliferation reached its peak after 3 days, as depicted in Figure 4(B). The doubling time of CRC (79.83 h) was lower compared with FC (84.68 h) ($p < 0.05$).

DISCUSSIONS

Currently, more than 300 clinical trials using hWJ-MSCs for therapeutic applications. However, a considerable challenge lies in developing a technique that can efficiently and cost effectively produce numerous high-quality cells to advance MSC-based clinical progress (Zhou et al. 2021). How cryorevival activity occurs in hWJ-MSCs must be understood to address this limitation. Early MSC-based clinical trials faced challenges, including the use of cryopreserved cells in immunotherapy studies. Cryobiology research has primarily focused on mitigating cellular damage during handling, particularly in addressing extreme dehydration, which can delay cell recovery (Chian 2010).

The clinical applications of MSCs require the sustained preservation of hWJ-MSC source features following cryopreservation, aiming to reduce cell recovery percentage. This experiment was designed to assess whether cryopreservation considerably affects isolated MSCs from Wharton's Jelly at the end of passage 3 and their sensitivity to stressful

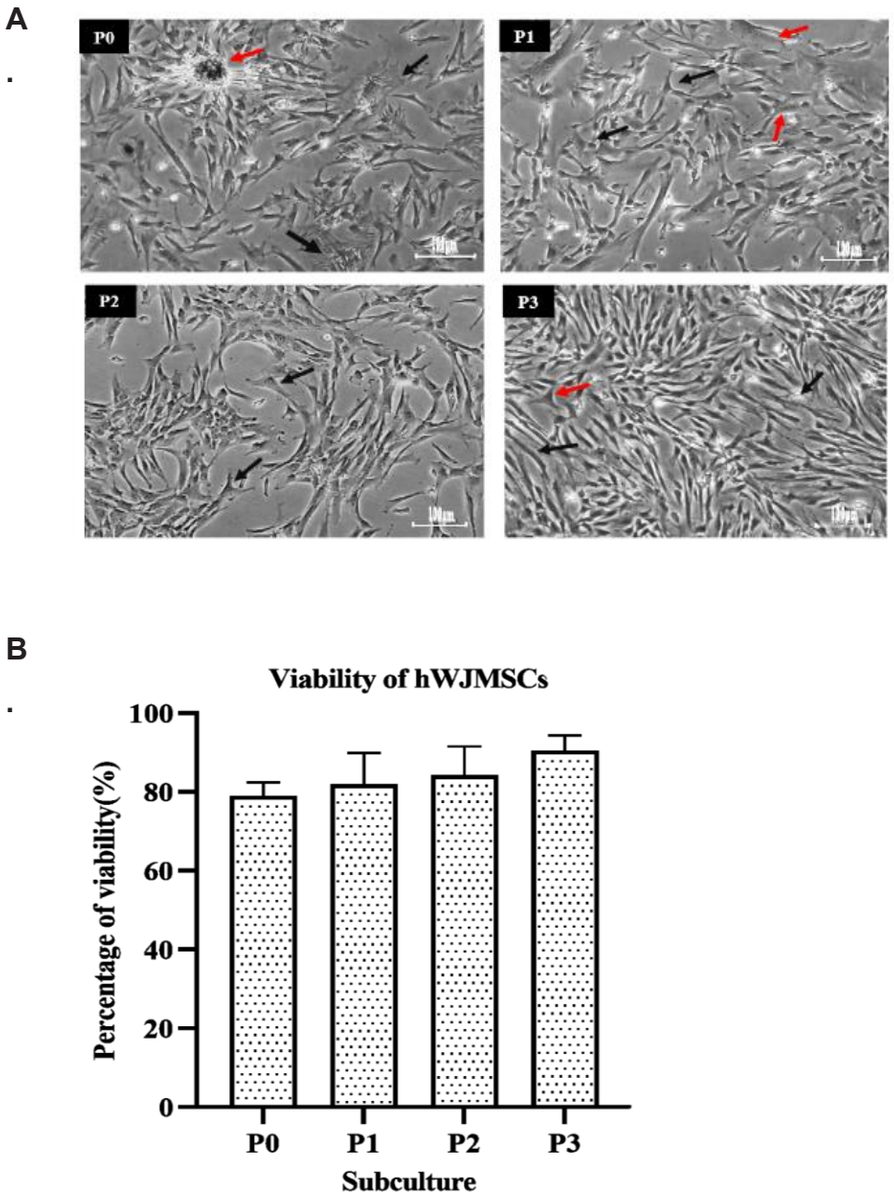

FIGURE 1. Initial morphology study and percentage of viability of primary hWJ-MSCs cell line. A. Adherent, thin to flat tripod/bipolar shaped spindle morphology (indicated with arrows) of hWJ-MSCs from passage 0 (P0) to passage 3 (P3) (n=4). magnification, x20. Bar scale, 100 µm. B. Viability percentage of hWJ-MSCsMSCs for each passage showed more than 70% from P0 to P3 (n = 4). Significant difference among subculture was considered $p < 0.05$

conditions during the immediate recovery period. Many studies on cryopreservation have explored various systems, including changes in freezing media, freezing temperature, and rapid cooling techniques like vitrification (Bahsoun, Coopman & Akam 2019).

A previous study yielded inconsistent outcomes regarding CRCs, highlighting that solely measuring cell viability can lead to false positive results (Murray & Gibson 2020). Therefore, the storage quality of hWJ-MSCs must be assessed with the goal of achieving an

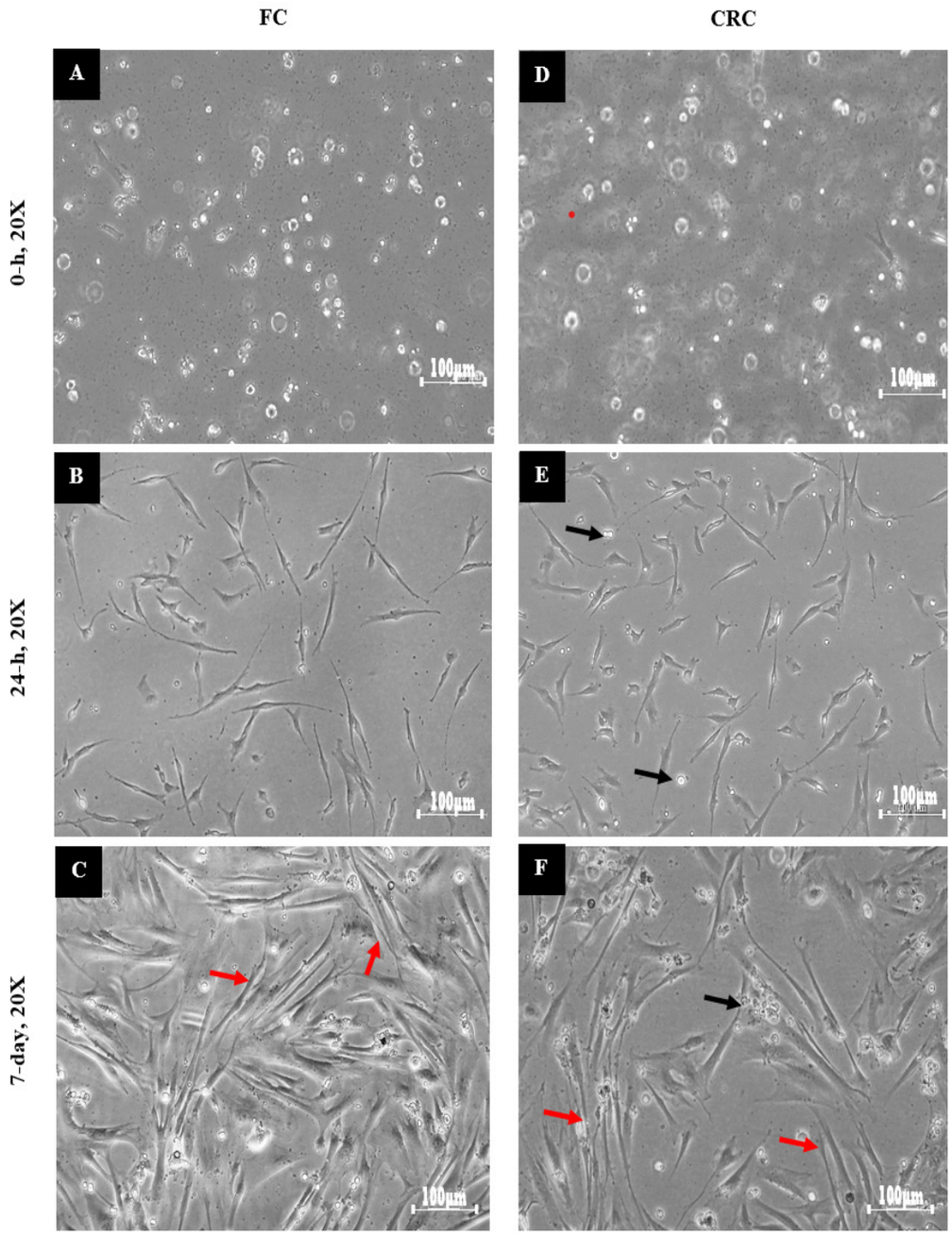

FIGURE 2. Comparison of morphology of fresh and cryopreserved cells. Cell morphology of FC (A,B,C) and CRC groups. (D,E,F) at passage 4. Cryo-revived cells showed presence of cell debris (E, F, black arrow). The cells became thinner spindle-shaped cells by Day 7 (C, F, red arrow). 20X magnification. Scale bar, 100 µm

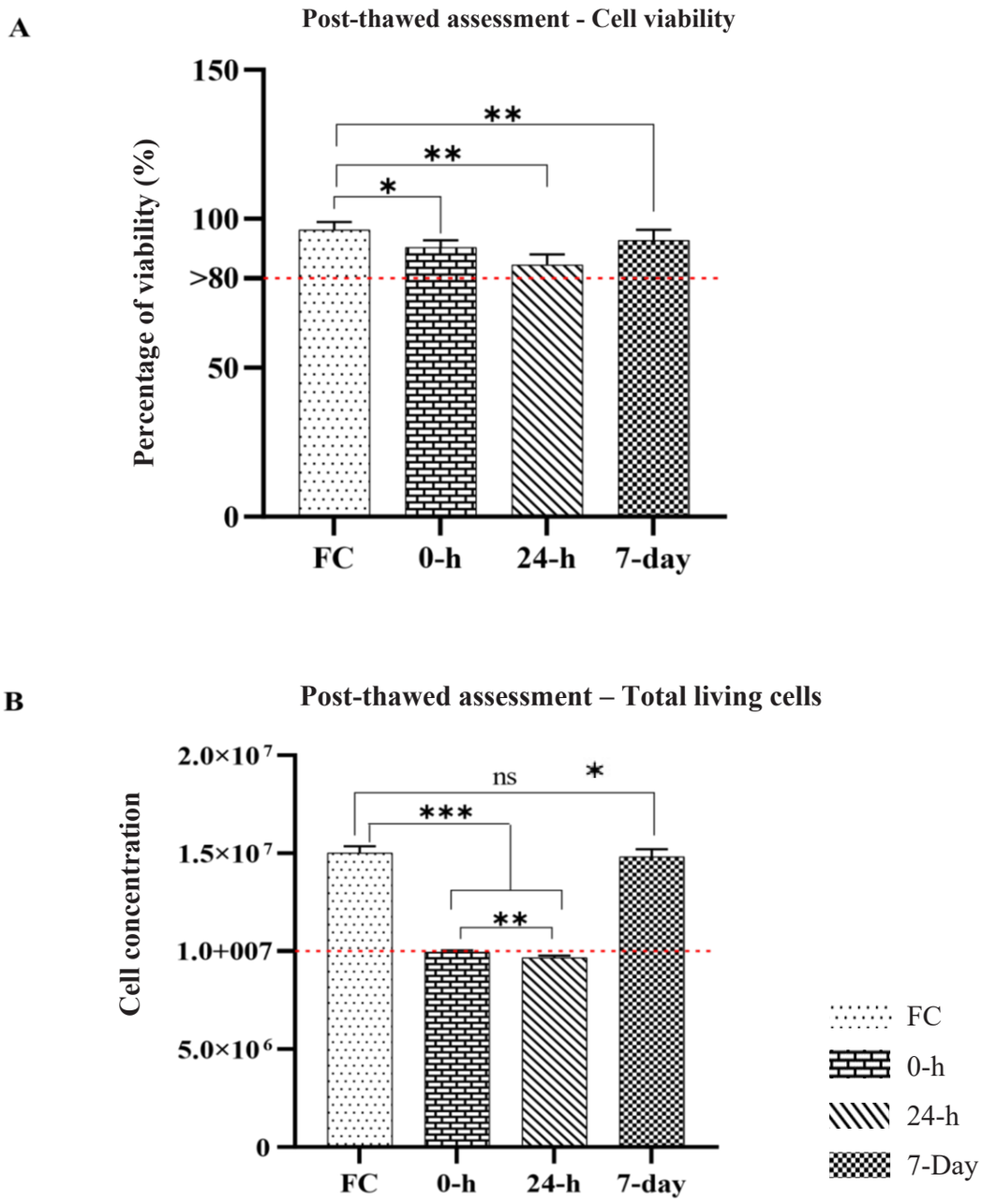

FIGURE 3. (A) Measurement of % cell viability of hWJ-MSCs for all group showed >80% viability. Statistically significant difference was showed between FC and CRC groups. (B) Total Living Cells after cryo-revival. Representative dotted line shows the initial total cells prepared, 1.0×10^7. Statistically significant difference was "*" showed between FC and CRC 7-day; "**" showed between CRC-0h and CRC 24-h. Statistical analysis done by 2-way ANOVA and two-tailed paired t-test

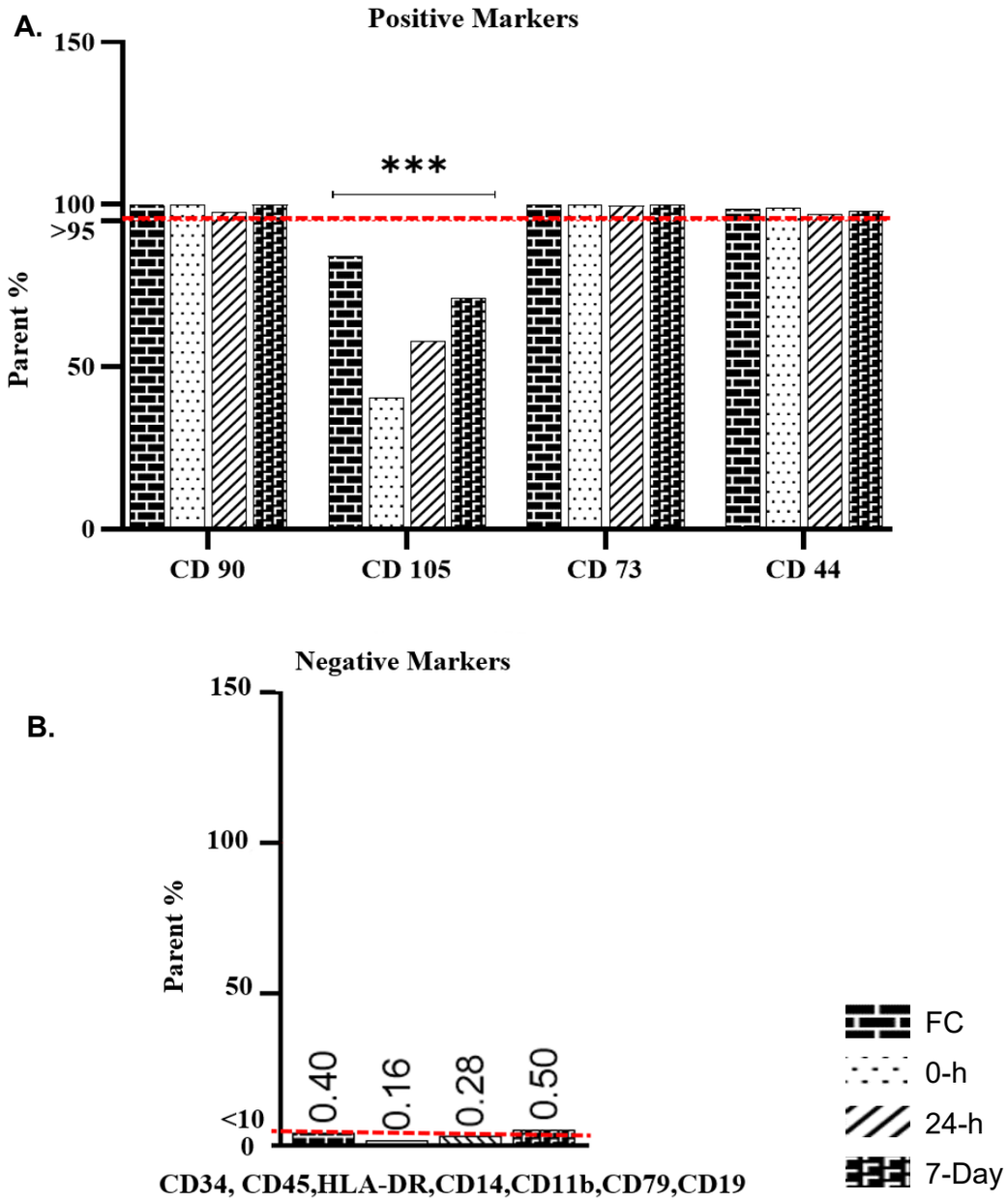

FIGURE 4. Flow cytometry analysis. (A) Numbers represent percentages of CD markers positive cells. The analysed positive markers were expressed in >95% of the cells except for CD105 (B) Negative markers were expressed

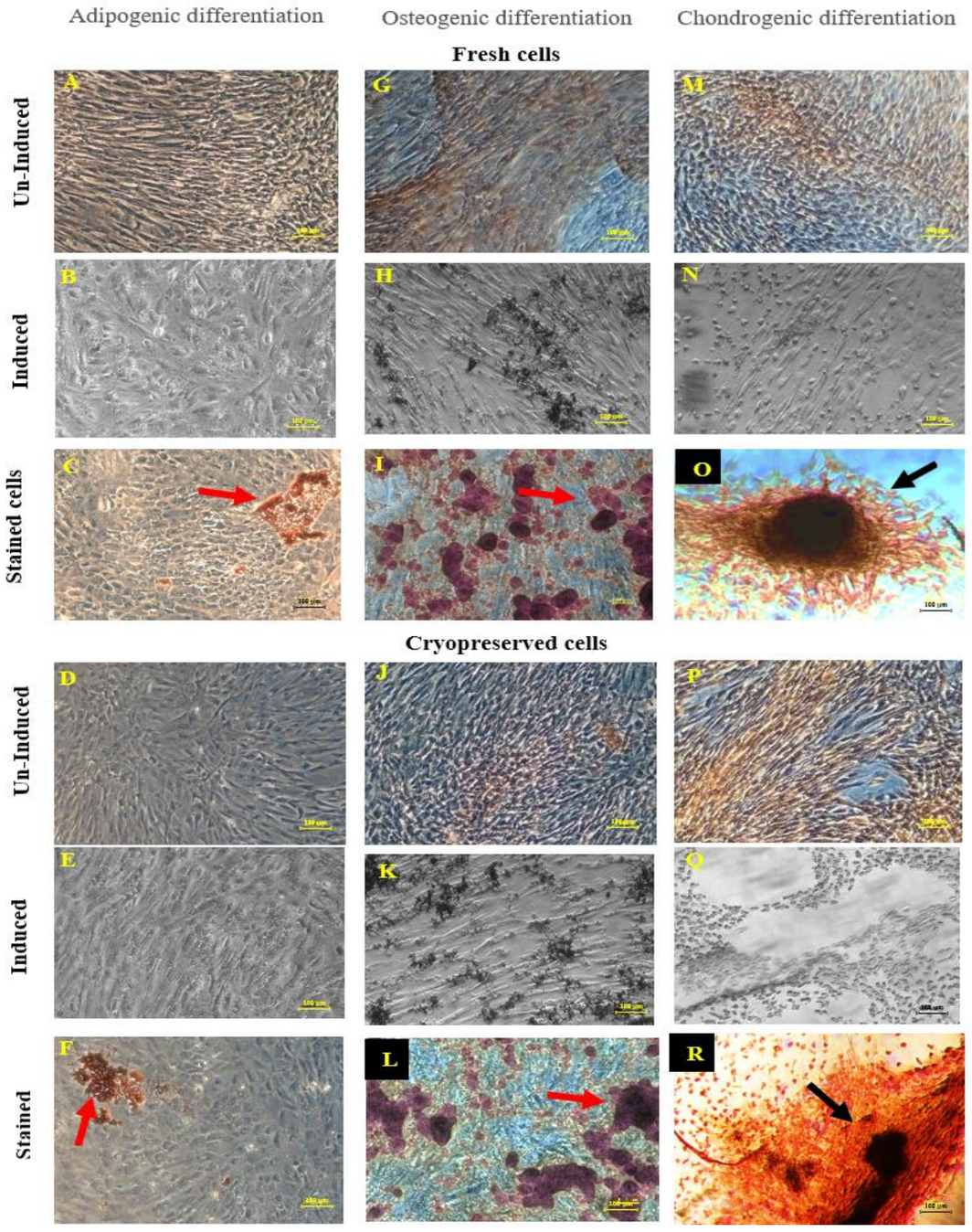

FIGURE 5. There were no observable differences found in trilineage differentiation between FC and CRC (40X) (n = 3). Oil red staining showed fat droplets (C, F, red arrow). Alizarin Red staining showed calcium deposit (I,L, red arrow). Chondrogenic-differentiated cells formed chondrocyte pellets that were stained by Safranin O (O, R, black arrow). Scale Bar, 100 µm

optimal recovery period whilst stabilising their viability and characteristics before therapeutic application. In this study, we conducted a comprehensive examination encompassing morphology, viability testing, and MSC characterisation of hWJ-MSCs to mitigate the risk of false positive outcomes during post-thaw recovery. To minimise false positives, we established a cutoff level of >80% in the cell viability test, as indicated by the dotted line in Figure 2(A), following the approach outlined by Naadjk et al. (2016). Numerous studies have assessed the post-thaw recovery at 0 h, 24 h and 7 day intervals and how the potency of hWJ-MSCs compared with that of fresh hWJ-MSCs (noncryopreserved cells).

Cryodamage typically has a limited effect on cell viability post thawing. In our experiment, we observed a significant reduction in cell viability and TNC for the CRC-0h and CRC-24h groups, except on the seventh day. The decrease in cell viability in CRC-24h may be attributed to a delayed apoptotic event, as suggested by previous studies (Pollock et al. 2017; Shivakumar et al. 2016). However, this phenomenon was not as evident in CRC-0h. The overestimation of CRC-0h results may be due to CPA molecular reactions, leading to reversed apoptosis, as reported by Bahsoun, Coopman and Akam (2019). Despite CRC-0h showing numerous living cells, these cells did not have sufficient time to attach to the flask's surface. According to Lechanteur et al. (2016), cryodamage to cells is transient, and cells return to normal behaviour after 24 h. To minimise ice recrystallisation and allow gradual removal of the CPA, a minimum of 24 h is recommended (Bharti et al. 2019; Whaley et al. 2021). Therefore, CRC-0h is not suitable for maintaining the stability and viability of hWJ-MSCs for immediate cell therapy.

Many approaches suggest choosing 24 h defrosted samples, which may provide good stability for cell-based products despite lower total cell recovery. Early apoptosis can be reversed by giving MSCs as little as 24 h to recover through cryorevival. Although CRC-24h cells adhered to the flask's bottom, they maintained a rounded shape and had not expanded into a spindle shape, indicating that they require more time to regain their MSC's multipotent characteristics. Abazari et al. (2017) demonstrated that MSCs cryorevived within 24–72 h still possess suitable properties. The cryoprotectant's effect is considerably overestimated in 0 h and 24 h cryorevived culture times, and viability tests may yield a high likelihood of false-positive results.

Remarkably, Marquez-Curtis et al. (2015) observed a higher cell count within 5–7 days of post-thaw recovery culture. The total number of living cells in hWJ-MSCsafter day 7 exhibited no significant difference compared with the FC group, suggesting that the cells continued to proliferate actively, following CRC's post-thawing recovery. The seven-day recovery period notably enhanced hWJ-MSC cell viability, as illustrated in Figure 3. Although CRC-7d displayed a slightly lower confluence of spindle-shaped and elongated cells compared to FC, both hWJ-MSC populations maintained their morphology even after cryopreservation. In summary, the effect of cryopreservation and the cell recovery period in this study only had a marginal effect on hWJ-MSCs' viability and the total number of living cells after 24 h, whereas CRC-7d demonstrated the most significant growth on the seventh day after seeding.

The CRC-0h population of parent cells appeared smaller than the other CRC groups, possibly indicating phenotype drift, as discussed by Murray and Gibson (2020). The levels of CD90, CD73, and CD44 were consistently above 95% (Figure 4.5, Section A), whereas negative markers, such as CD34, CD45, HLA-DR, CD14 or CD11b, CD79 and CD19 ranged from 0.3% to 0.7%. These markers did not significantly differ in all CRC groups compared with the FC group. Although CRC-24h showed lower cell viability and TNC compared with the initially aliquoted cell amount, results suggests that hWJ-MSCs gradually express their MSC markers. However, the number of cells expressing CD105 was significantly reduced in FC and CRC groups. According to Antebi et al. (2019) and Bahsoun, Coopman and Akam (2019), no significant differences were observed in marker expression after cryorevival. CD105 expression is typically associated with strong immunomodulation capacity for the chondrogenesis of MSCs (Antebi et al. 2019; Bahsoun, Coopman & Akam 2019; Hieu Pham, Bich Vu & Van Pham 2019; Hieu et al. 2019). However, in this study, CD105 expression did not appear to affect the stemness or differentiation of MSCs in FC and CRC groups.

Furthermore, the CD105 outcomes showed that protein levels gradually increased from 0 h to 7 days (Figure 3(A)). Francois et al. (2012) reported that a 24 h cryorevival period was sufficient to restore the immunophenotype properties of cryorevived hWJ-MSCs to a level similar to that of MSCs cultured for 7 days (Francois et al. 2012). In a previous study by Brohlin et al. (2017), hBM-MSCs exhibited reduced CD105 expression in serum-free medium, but this has not been previously reported for hWJMSCs. A similar result was shown by Dominici et al. (2006) and a decrease in the

percentage of CD105 expression was also noted by Qu et al. (2020). In our study, no apparent effect of FBS on CD105 expression in culture media was observed. This result may be attributed to either high exposure to the freezing medium or the possibility of a change in the cell surface marker profile (Qu et al. 2020).

Further *in vivo* experiments are necessary to confirm the cells' differentiation ability after being cultured in an FBS medium. However, conflicting results regarding the effects of CD105 on the trilineage differentiation capacity of BM-MSCs or ASCs have been reported (Cleary et al. 2016; Levi et al. 2011). hWJMSCs retained their positive expression of CD markers (CD90, CD73 and CD44) even after cryopreservation, indicating that the cell recovery period had no considerable effect on the integrity of the cells. Although FC exhibited optimal cell counts, it significantly reduced the expression of CD105. Furthermore, the FC group did not meet the expected range guided by the ISCT (Kannaiyan et al. 2017).

In addition, assessing multilineage differentiation potential is crucial to determining MSC characteristics. The multipotency of these cryopreserved hWJ-MSCs was evaluated by inducing them to differentiate into osteogenic, adipogenic, chondrogenic and neural lineages. However, the flow cytometry immune profiling capability to express surface markers and differentiation potential was limited to cell passages 3–5, as described by Ali et al. (2015). As depicted in Figure 4.7, images G and L showed deposits in osteogenically induced cryo-hWJ-MSCs when stained with Alizarin red stain, similar to fresh hWJ-MSCs, indicating that these cells, after cryopreservation, retained their osteogenic potential after 21 days of osteogenic stimulation. The deposits indicate that calcium accumulation was observed after two weeks, appearing as small, round Alizarin red-positive nodules in the cells.

Furthermore, cryopreserved hWJ-MSCs were induced to follow an adipogenic lineage exhibited positive staining for the oil red O stain, confirming the formation of lipid droplets in these induced cells (Figure 4.7, Image F), thus, verifying their differentiation capability into an adipogenic lineage, as previously demonstrated. In the inducing culture medium, no noticeable changes in the morphology of the adipogenic differentiation cells were observed. After a few days, hWJ-MSCs displayed small lipid droplets and vacuole-like structures in the cytoplasm, as indicated by oil-red staining (Chen et al. 2021). By contrast, control unstained cells did not display any fat droplets. Subsequently, we assessed the chondrogenic differentiation ability of cryo-hWJMSCs after the induction period. These cryopreserved hWJMSCs exhibited positive staining for Safranin O in expanded collagen fibres but not in chondrocyte pellets, with no observable changes in undifferentiated cells (Yong et al. 2015). In our study, the trilineage differentiation of hWJ-MSCs showed no apparent difference after the cells were revived in the FBS medium, despite the markedly different CD105 levels. This finding aligns with a recent study that used a similar procedure and found that our cryopreserved cells retained their stem cell function (Omar et al. 2019; Shivakumar et al. 2015; Yi et al. 2020).

The growth curve exhibited significant differences in the hWJ-MSC CRC group between days 1, 3, 5, 7 and 9 in terms of viability and doubling time. FBS concentration inhibited CPA molecular activity starting from day 3 onwards, resulting in a substantial improvement in CRC viability (Bharti et al. 2019), with the ability to maintain high levels of doubling time up to day 3. However, reducing the CPA concentration did not affect post-thaw viability (Tripathy 2017). Notably, improved subconfluent cryorevived hWJ-MSCs were obtained when 10% FBS was replaced, and the cryoprotectant solution was withdrawn, leading to the observed growth curve. The ideal post-thaw viability was achieved, with a total of 8.7×10^3 cells/mL on day 9, compared with the initial cell count of 1×10^3 cells/mL on day 1. According to Figure 6(A), cryorevival on day 7 exhibited a logarithmic growth phase, and the CRC maintained viable cells until day 9. By contrast, the CRC group displayed a similar doubling period as the FC group. For each group, the starting seeding density was roughly 1×10^3 cells/well. The results indicate that from day 1 to day 9, the cell growth rate increased. We hypothesised that superior quality, as measured by a higher percentage of living cells in the post-thaw period, would determine the outcome of CRC-7d, as evaluated against the FC group.

LIMITATIONS OF THE RESEARCH AND SUGGESTIONS FOR FUTURE STUDIES

Whilst numerous studies have indicated that the cryopreservation effect is negligible in short-term and long-term cell-based product manufacturing, limitations persist in certain clinical research areas. Comprehensive research has highlighted the necessity of CPAs to mitigate cell damage during freezing. Future studies should explore the extent to which CPA concentration is reduced in intracellular CRC hWJ-MSCs *in vivo*, and

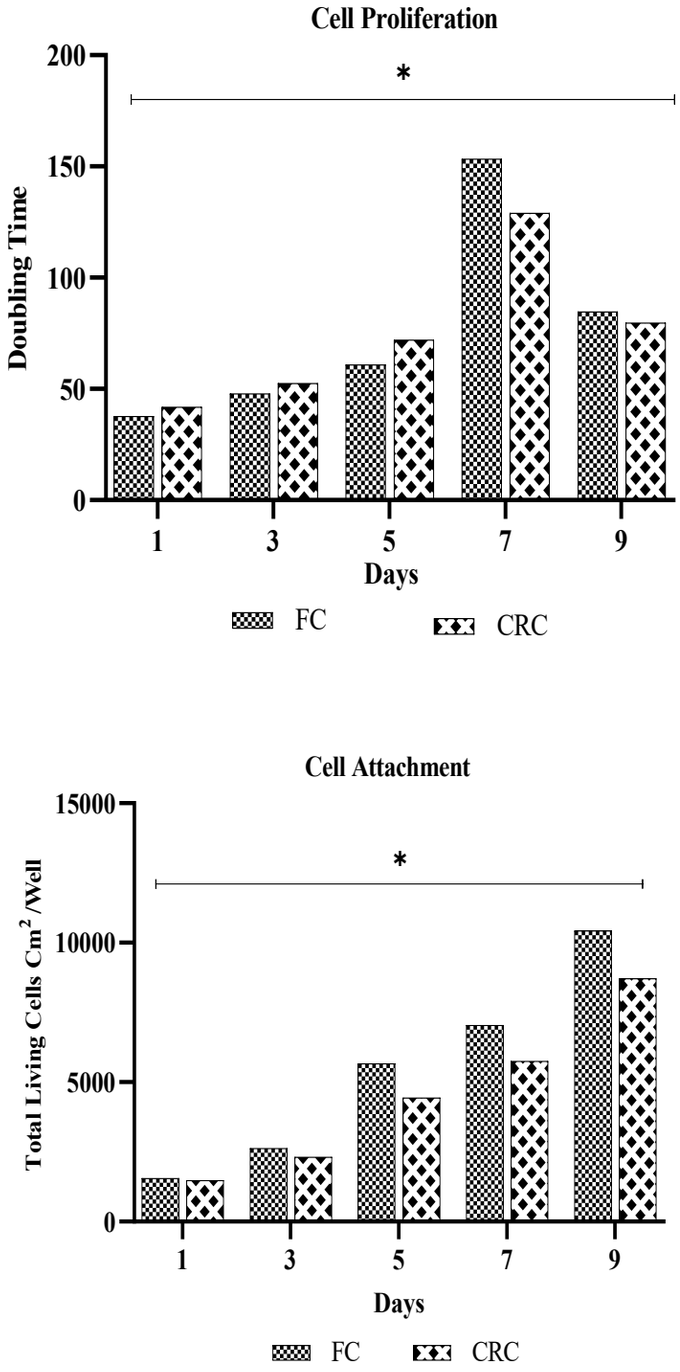

FIGURE 6. The growth curve of FC and CRC until day 1-9 (n=3) P value *<0.05 for both doubling time (A) and total living cells (B)

tests are essential to confirm the results of cell revival properties and function. However, several limitations remain, including low cell concentration, the choice of culture medium, the duration of cell attachment and the potential for regrowth. Additionally, the duration of a cell's revival can affect cellular properties, and the low viability of cryopreserved cells can result in poor immunophenotypes. Cells can maintain the expression of specific proteins even as cell numbers and viability decrease, as long as viability remains above 60%.

Notably, CPA and FBS have a considerable effect on the cell viability of CRC, potentially affecting MSC CD expression. Therefore, the use of CPA, serum and cell concentration during cell growth must be monitored before cryopreservation. Concerns about their effects on cryopreserved cells are necessary to minimise complications and optimise the post-thaw recovery period. A previous study has suggested that the growth medium may lead to lower expression of CD105 markers and impaired therapy outcomes. However, this study found that hWJ-MSCs retained their potency and differentiated into MSC lineages, indicating that CRC could preserve cell characteristics even after the freeze/thaw cycle.

Moreover, future research should aim to gain an improved understanding of cell behaviour during the cryorevived period to develop safer and more effective cryopreservation methods. Studying cell behaviour can provide insights into the production and structure of extracellular matrix-like components through various scaffolds. Scaffold design has become integral to tissue engineering, aiding in the study of annexin cell attachment and the migration of living cells. The parameters to investigate should include cell viability using a flow cytometer and the total number of dead cells using an Annexin V assay. These assays can help determine whether the low expression of CD markers is due to the accumulation of dead cells or the effects of serum or CPA on marker purity.

Furthermore, cell recovery rates must be measured through cell counting and post-thaw monitoring to prevent the spread of false positives in this evolving field. Measuring cell recovery rates using techniques, such as a trypan blue exclusion assay can help standardise cryoprotectant assessment. Cryopreservation outcomes are currently measured using two main methods: cell viability and the ratio of live cells to total cells post thaw. This discrepancy highlights the need for a standardised approach to evaluating cryoprotective effects, especially given the variations in cell survival amongst different cell lines or primary cells.

Additionally, the detection of apoptosis is an effective technique for assessing cell viability because it can identify cells undergoing apoptosis based on cell membrane integrity, DNA fragmentation, mitochondrial membrane potential loss and mitochondrial permeabilisation. Future studies should consider incorporating these methods to obtain a comprehensive assessment of cryopreservation outcomes and ensure the reliability and reproducibility of results in the field of regenerative medicine.

Conclusions

Cryopreservation offers the advantage of making MSCs readily available for immediate therapeutic use, eliminating the need to wait for cells. However, our study identified certain limitations in the cryopreservation methods applied to hWJ-MSCs, particularly concerning the time it takes for them to regain their spindle shape and reach the desired level of confluency for optimal viability. Nonetheless, the cells could adhere to the surface after 24 h. Whilst all positive markers of hWJ-MSCs in the CRC groups maintained their immunophenotypic features, reduction in CD105 expression across all groups was consistent. This reduction in viability and marker expression could be attributed to the influence of CPA and FBS.

Despite these challenges, our study demonstrated that hWJ-MSCs retained their potential to differentiate into various MSC lineages. This result suggests that CRC was successful in preserving the fundamental characteristics of these cells even after cryopreservation. The study also highlighted the importance of allowing CRC hWJ-MSCs at least 7 days to stabilise their expression of multipotent markers. Moving forward, further investigations are warranted to explore additional disease indications and delivery routes, aiming to gain a comprehensive understanding of the conditions and modes of infusion for hWJ-MSCs within a few hours post thaw. This study contributes to the optimisation of hWJ-MSC-based therapies.

Acknowledgements

This study was funded by the Faculty of Medicine, UKM (FF-2020-386/1) and Da Vinci Healife Sdn. Bhd (FF-2020-386). The study was approved by Faculty of Health Science from UNISEL (J160364E) and UKM research ethics committee (PPI/111/8/JEP-2020-506).

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