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Human Wharton's Jelly Stem Cell Conditioned Medium Enhances Freeze-Thaw Survival and Expansion of Cryopreserved CD34+ Cells

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Abstract Hematopoietic stem cells (HSCs) from umbilical cord blood have been successfully used to treat blood disorders but one major hurdle is the relatively low cell dose available. Double cord blood unit transplantation results in elevated engraftment failure because one unit predominates over the other. Various approaches are thus being undertaken to expand HSCs ex vivo from single cord blood units. We report here a protocol involving slow freezing (−1 °C per minute to −120 °C) + freezing medium containing DMSO + FBS + 24 h-50 % hWJSC-CM that enhances thaw-survival of CD34+ cells. Post-thawing, the fold, percentage and colony forming unit numbers of CD34+ cells were significantly increased (2.08±0.3; 102±1.17 %; 1.07±0.02 respectively) while the percentages of apoptotic, necrotic, dead and sub-G1 phase cells (91.06±3.63 %; 91.80±5.01 %; 95.6±3.61 %; 86.1±16.26 % respectively) were significantly decreased compared to controls. Post-thaw culture in 24 h-50 % hWJSC-CM+FBS for 72 h showed further significant increases in CD34+ cells (fold: 2.28±0.17; percentage: 153.3±21.99 %, CFU: 1.6±0.19) and significant decreases in apoptotic, necrotic, dead and sub-G1 cells (49.2±3.59 %; 62.0±4.30 %; 56.6±5.06 %; 28.6±5.74 % respectively) compared to controls. We hypothesize that these improvements are probably related to the high levels of cytokines, cell adhesion molecules and growth factors in hWJSC-CM that help to preserve cell membrane integrity during freezing and stimulate mitosis post-thaw. A 24 h-50 % hJWSC-CM may be a useful supplement for freezing CD34+ cells in cord blood banks.

Keywords Cord blood banks · Expansion of human umbilical cord blood CD34+ cells · Human Wharton's jelly stem cell conditioned medium · Slow freezing

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Introduction

Stem cells hold tremendous promise for the treatment of various incurable diseases by transplantation therapy. Of the various stem cell types, hematopoietic stem cell (HSC) transplantation has been the most successful in the clinic. Since the introduction of bone marrow HSC transplantation in 1968, HSCs have been used in the treatment of hematopoietic diseases such as leukemia, lymphoma, thalassemia and autoimmune disorders. HSC transplantation has also been shown to produce promising results for the treatment of chronic liver failure and acquired immunodeficiency syndrome [1, 2].

HSCs are obtained from many different sources such as bone marrow (BM), peripheral blood (PB) and umbilical cord blood (UCB). HSCs isolated from the UCB have several advantages for transplantation therapy compared to the BM and PB [3–5]. They are easily collected and stored in cord blood banks, have lesser risk of graft versus host disease (GVHD) in transplant recipients due to their immune naivety and require less stringent criteria for donor-recipient matching. Additionally they have high proliferation rates, autocrine production of hematopoietic factors and longer telomere lengths due to their younger chronological age.

However, a major limitation to their use in transplantation is the low cell numbers in a single UCB unit. The yield of

HSCs from a single freeze-thawed UCB unit is typically about 1.0×10^7 cells [6]. This number is far less than the recommended cell numbers required for transplantation. Recommended cell numbers range from 2.5 to 5×10^6 CD34+ cells/kg for a successful engraftment [7, 8]. As such, single unit UCB-derived HSC transplantation is often a challenge for the treatment of adults [6]. Two of the strategies for successful transplantation in adult humans are (i) the use of double UCB unit transplantation and (ii) ex vivo expansion of the single UCB unit. However, double UCB transplantation has been associated with delayed engraftment and elevated engraftment failure and usually one UCB unit ultimately predominates over the other [9]. Since there is a threshold dose below which cord blood transplantation recipients have engraftment failure, the ex vivo expansion of HSC numbers from single UCB units for the treatment of adults is a more plausible approach to successful transplantation therapy. Thus, several approaches are being attempted to increase HSC numbers in single UCB units. One approach is the refinement of existing cryopreservation protocols so as to increase freeze-thaw survival cell numbers.

For most types of cells, slow programmed freezing gives optimum freeze-thaw survival results compared to rapid or ultra-rapid methods because of slow ice crystal formation within the cells helping to preserve them. However, existing slow freezing protocols for HSCs are far from ideal as there is significant cell death and loss of CD34+ cell populations after freeze-thaw [10]. This is because the slow freezing process results in nutrient deprivation, cellular dehydration, stress changes to structure, and physiology of HSCs. Hence the post-thaw survival rates are often compromised [11]. Cryoinjury-induced apoptosis has also been reported as one of the main reasons for the loss of viability after cryopreservation [12]. Post-thaw apoptosis of CD34+ cells has also been shown to affect the recovery of viable CD34+ cells required for transplantation therapy [13, 14]. Stroh et al. [15] reported that the post-thaw apoptosis of CD34+ cells was mediated by the mitochondria and activated caspase 3 activity. Previous studies used catalase and trehalose additives to provide cryoprotection by preventing apoptotic cell death [16]. The ROCK inhibitor Y-27632 was shown to enhance post-thaw survival and recovery of human embryonic stem cells and induced pluripotent stem cells [17], human mesenchymal stem cells (hMSCs) [12] and human umbilical cord Wharton's Jelly stem cells (hWJSCs) [18], but on the contrary it was shown to impair the survival, recovery and expansion of cryopreserved CD34+ cells [10]. Currently, novel methods are being explored to improve the freeze-thaw viability and numbers of cryopreserved UCB-HSCs [5]. An optimal protocol for cryopreservation of HSCs that could overcome cryopreservation-induced damage needs to be developed to support HSC transplantation [19].

We and others have reported that hWJSCs have several beneficial advantages over other mesenchymal stem cells (MSCs) not only for cell based therapies, but also as an anticancer agent and as stromal support for HSC expansion [20–23]. We also showed that the secretions in the culture medium conditioned by hWJSCs over 24–72 h had potent molecules that had strong anticancer effects and also supported the expansion of HSCs ex vivo [22]. When UCB-HSCs were exposed to hWJSC conditioned medium (hWJSC-CM) they immediately became motile, put out pseudopodia-like projections and underwent proliferation. With time in culture, increased CD34+ cell numbers and colony forming units were produced which eventually generated all the lineages of normal hematopoiesis [22]. A proteomic analysis of the hWJSC-CM showed high concentrations of several families of important agents such as interleukins, growth factors, glycosaminoglycans and cell adhesion molecules. We attributed the spontaneous activity and expansion of the HSCs to these bioactive soluble molecules in the hWJSC-CM [22]. Given the potent actions and potential therapeutic value of the agents in hWJSC-CM we explored whether the incorporation of hWJSC-CM as a supplement in freezing and thawing media in cryopreservation protocols and in post-thaw culture would enhance freeze-thaw survival and subsequent increase of CD34+ cell numbers.

Materials

Propagation of hWJSCs and Preparation of hWJSC-CM

1. Human umbilical cords were obtained after informed patient consent and institutional domain specific review board (DSRB) ethical approval. Human Wharton jelly stem cells were derived from the umbilical cords using the method of Fong et al. [24]. [See Note 1].
2. T75 tissue culture flasks (Thermo Fisher Scientific, Rochester, NY).
3. hWJSC medium [80 % DMEM (high glucose) medium supplemented with 20 % fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 1 % non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 % insulin-transferrin-selenium (ITS), antibiotic/antimycotic mixture (50 IU penicillin, 50 μg/ml streptomycin) (Invitrogen Life Technologies, Carlsbad, CA) and 16 ng/ml basic fibroblast growth factor (Millipore Bioscience Research Agents, Temecula, CA)].
4. Incubator (37 °C, 5 % CO_2 in air).
5. Sorvall Lengend RT + Centrifuge (Thermo Fisher Scientific, Rochester, NY).
6. StemSpan Serum Free Expansion medium (StemSpan SFEM, Stem Cell Technologies, Vancouver, BC) supplemented with 2 mM L-glutamine, 1 % antibiotic/antimycotic mixture (Invitrogen Life Technologies, Carlsbad, CA).

7. 0.22 μM filters (Millipore Bioscience Research Agents, Temecula, CA).

Freezing and Thawing

1. Freezing medium (Experimental) (FME) [24 h-50 % hWJSC-CM supplemented with 2 mML- glutamine, 1 % antibiotic/antimyotic mixture (Invitrogen Life Technologies, Carlsbad, CA), 40 % FBS (HyClone Thermo Scientific, Rochester, NY) and 10 % DMSO (Sinopharm Chemical Reagent Co Ltd, Shanghai, China)]
2. Freezing Medium (Control) (FMC) [50 % StemSpan SFEM (StemCell Technologies Vancouver, BC) supplemented with 2 mML-glutamine, 1 % antibiotic/antimyotic mixture (Invitrogen Life Technologies, Carlsbad, CA), 40 % FBS (HyClone Thermo Fisher Scientific, Rochester, NY) and 10 % DMSO (Sinopharm Chemical Reagent Co.Ltd, Shanghai, China)]
3. 1 mL cryovials (Thermo Fisher Scientific, Rochester, NY).
4. Controlled rate freezer (Planer Kryo10 Series II, Planer PLC, London, UK).
5. Liquid nitrogen tank
6. Liquid nitrogen
7. Water bath
8. Thawing medium (TM) [IMDM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 20 % FBS (HyClone Thermo Fisher Scientific, Rochester, NY), 2 mML-glutamine, 1 % antibiotic/antimyotic mixture (Invitrogen Life Technologies, Carlsbad, CA)]
9. Post-thaw medium (Experimental) (PTME) [24 h-50 % hWJSC-CM supplemented with 20 % FBS (HyClone Thermo Fisher Scientific, Rochester, NY), 2 mML-glutamine and 1 % antibiotic/antimyotic mixture (Invitrogen Life Technologies, Carlsbad, CA)]
10. Post-thaw medium (Control) (PTMC) ([StemSpan SFEM supplemented with 20 % FBS (HyClone Thermo Fisher Scientific, Rochester, NY), 2 mML-glutamine and 1 % antibiotic/antimyotic mixture (Invitrogen Life Technologies, Carlsbad, CA]
11. 24 well plates (Thermo Fisher Scientific, Rochester, NY).
12. Incubator (37 °C, 5 % CO_2 in air)

Expansion of UCB CD34+ Cells

1. Human umbilical cord blood (UCB) CD34+ HSCs were purchased as kits from Stem Cell Technologies, Vancouver, BC. Ethical approval for their purchase and use was given by the National University of Singapore Institutional Review Board (NUS-IRB).
2. StemSpan SFEM (StemCell Technologies Vancouver, BC, Canada).
3. CC110 Cytokines Cocktail (StemCell Technologies Vancouver, BC, Canada)
4. 60 mm dishes [Becton Dickson (BD), Franklin Lanes, NJ]
5. Incubator (37 °C, 5 % CO_2 in air)

Cell Morphology

1. Olympus IX70 Inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan)

MTT Cell Proliferation Assay

1. 96 well plate (Thermo Fisher Scientific, Rochester, NY).
2. StemSpan SFEM (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 20 % FBS (HyClone ThermoScientific, Rochester, NY), 2 mML-glutamine and 1 % antibiotic/antimyotic mixture (Invitrogen Life Technologies, Carlsbad, CA)
3. MTT kit [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, (MTT) 0.5 mg/ml (Duchefa Biochemie B.V., Haarlem, Netherlands)
4. Incubator (37 °C, 5 % CO_2 in air)
5. Dimethylsulfoxide (DMSO) (Sinopharm Chemical Reagent Co.Ltd, Shanghai, China)
6. Microplate ELISA reader (mQuant, BioTek and Winooski, VT, USA)

Annexin V-FITC/PI Assay

1. Phosphate buffered saline without calcium and magnesium [PBS(-)](Invitrogen Life Technologies, Carlsbad, CA)
2. Annexin V binding buffer (10×) (BioVision, Inc., Mountain View, CA)
3. Annexin V-FITC (BioVision, Inc, Mountain View, CA)
4. Propidium iodide (PI) (1 μg/mL) (Invitrogen Life Technologies, Carlsbad, CA)
5. 40 μM nylon strainer [Becton Dickson (BD), Franklin Lanes, NJ]
6. CyAn™ ADP Analyser (Beckman Coulter, Fullerton, CA)

Live/Dead® Assay

1. PBS(-) (Invitrogen Life Technologies, Carlsbad, CA)
2. Live/Dead® Viability/Cytotoxicity kit for mammalian cells (Invitrogen Life Technologies, Carlsbad, CA)
3. 40 μM nylon strainer (Becton Dickson (BD), Franklin Lanes, NJ)
4. CyAn™ ADP Analyser (Beckman Coulter, Fullerton, CA)

Cell Cycle Assay

1. 70 % ethanol
2. PBS(-)
3. Propidium iodide (PI) (20 μg/mL) (Invitrogen Life Technologies, Carlsbad, CA)

4. RNase A (100 μg/mL) (AppliChem GmbH, Garmstadt, Germany)
5. 40 μM nylon strainer [Becton Dickson (BD), Franklin Lanes, NJ]
6. CyAn™ ADP Analyser (Beckman Coulter, Fullerton, CA)

CD34+ Analysis

1 10 % normal goat serum (Invitrogen Life Technologies, Carlsbad, CA)
2 Primary anti-human CD34 antibodies (1:100) (Biolegends, San Diego, USA)
3 Secondary anti-mouse IgG (H+L) Alexa Fluor® 488 secondary antibody (1:750) (Invitrogen Life Technologies, Carlsbad, CA)
4 40 μM nylon strainer [Becton Dickson (BD), Franklin Lanes, NJ]
5 CyAn™ ADP Analyser (Beckman Coulter, Fullerton, CA)

Colony Forming Unit (CFU) Assay

1. IMDM supplemented with 20 % FBS, L-glutamine and antibiotic/antimyotic mixture (Invitrogen Life Technologies, Carlsbad, CA).
2. MethoCult™ H4435 medium (Stem Cell Technologies, Vancouver, BC)
3. 32 G blunt end needle (Stem Cell Technologies, Vancouver, BC)
4. 24 well plates (Thermo Fisher Scientific, Rochester, NY).
5. Inverted phase contrast microscope (Nikon Instruments, Tokyo, Japan).
6. Atlas of Hematopoietic Colonies from Cord Blood (Stem Cell Technologies, Vancouver, BC)
7. CytoSelect™Hematopoietic Colony Forming Assay (Cell Biolabs, Inc., San Diego, CA).
8. 96 well assay plate (Thermo Fisher Scientific, Rochester, NY).
9. TECAN GENios (TECAN Austria GmBH, Salzburg, Austria)

Methods

Propagation of hWJSCs and Preparation of hWJSC-CM

1. hWJSCs were grown in a T75 tissue culture flask with hWJSC medium until 70 % confluence as previously described [24] [See Note 2].
2. hWJSC-CM was prepared as previously described [22].
3. hWJSC medium was replaced with StemSpan SFEM medium supplemented with 2 mM L-glutamine and antibiotic/antimyotic mixture.
4. hWJSCs were cultured in this medium for 24 h, separated and filter-sterilized using a 0.22 μM filter.
5. hWJSC-CM was stored at -20 °C until use.
6. Preparation of hWJSC-CM was carried out on early hWJSC passages (3P to 5P).

Expansion of Commercial UCB CD34+ Cells

1 Flow Activated Cell Sorting (FACS) profile given by the company showed that more than 90 % of the cells were CD34+.
2 Expansion of the frozen UCB CD34+ cells was according to the manufacturer's instructions supplied with the kit.
3 The frozen cells in the cryovial were thawed within 1–2 min using a water bath (37 °C).
4 6 ml of thawing medium was slowly added drop by drop to the cryovial to prevent osmotic shock to the CD 34+ cells.
5 The cryovials were then centrifuged, supernatant decanted and cells resuspended in StemSpan SFEM medium supplemented with 2 mM L-glutamine, 1 % antibiotic/antimyotic mixture and cytokines cocktail CC110.
6 UCB CD34+ cells were first equilibrated and grown in this Expansion medium for 3 days before use for freeze-thaw survival experiments.

Freezing and Thawing of UCB CD34+ Cells

1 UCB CD34+ cells were frozen using slow programmed freezing in a controlled rate freezing machine (Kryo10 Series II) from room temperature at a freezing rate of −1 °C per minute to −120 °C as previously described [25]. Three different UCB samples were evaluated. Each UCB sample was divided into two groups A and B. Group A: Freezing Medium (Control) (FMC); Group B: Freezing medium (Experimental) (FME) [See Note 3]
2 The cryovials were removed at −120 °C and plunged into liquid nitrogen (−196°C) in a tank for long-term storage.
3 Cells were maintained at −196°C for at least 2 weeks before analysis.
4 Frozen CD34+ cells from Groups A and B were thawed rapidly within 1–2 min in a 37 °C water bath.
5 3 mL of thawing medium was then added into each cryovial and the cryovial centrifuged at 500 × g for 5 min.
6 The supernatant was then removed and the cell pellets from each of Groups A and B were divided into 2 Subgroups, with each Subgroup having Experimental and Control arms. Subgroup 1: *0 h post- thaw analysis* (Analysis done immediately after thawing) (Experimental arm: hWJSC-CM; Control arm: control). Subgroup 2: *72 h post-thaw analysis* (Analysis done 72 h after thawing) (Experimental arm: Thawed cells grown for 72 h in hWJSC-CM; Control arm: Thawed cells grown for 72 h in control medium).

7 The analyses included cell morphology, cell proliferation assay, CD34+ cell analysis, CFU assay, Annexin V-FITC/PI assay, Live/Dead® Viability assay and Cell Cycle assay.
8 Results were expressed as mean ± SEM and statistically significant differences between groups were calculated using the two-tailed Student's *t*-test using SPSS Statistic v 17.0 (SPSS, Inc, IL). A p value of <0.05 was considered as statistically significant.

Cell Morphology

1. Cell pellets from Experimental and Control arms were placed in their respective media and the cell morphology immediately observed.
2. No distinct morphological differences were observed in the UCB CD34+ cells between the two arms with cells in both arms showing their characteristic spherical shape and size [Fig. 1(a, b)].
3. Thawed cells from Experimental and Control arms were cultured for 72 h in their respective media and morphology observed.
4. More cellular debris was observed after 72 h culture in the Control compared to the Experimental arms [Fig. 1(c–f)]

Cell Proliferation Assay

1. Cell pellets from Experimental and Control groups were re-suspended in 100 μL StemSpan SFEM supplemented with 20 % FBS, L-glutamine and antibiotic/antimycotic mixture.
2. 10 μl of MTT (final concentration of 0.5 mg/ml) was added to each sample and incubated at 37 °C in a 5 % CO_2 in air atmosphere overnight.
3. The samples were then centrifuged at 1,000 × g for 5 min.
4. The supernatants were then removed and each cell pellet resuspended in 100 μl of DMSO. The cell suspensions were then incubated in the dark for 10 min.
5. Each sample was dispensed into the wells of a 96 well plate and the intensity of the purple colour was measured at 570 nm absorbance against a reference wavelength of 630 nm using a microplate ELISA reader.
6. A significantly greater number of CD34+ cells survived in hWJSC-CM compared to controls immediately after thawing (2.08±0.3 fold increase) [Fig. 2(a)].
7. Significantly greater proliferation rates of CD34+ cells were observed when they were frozen in hWJSC-CM and cultured after thawing for 72 h in hWJSC-CM compared to controls.
8. The mean ± SEM fold increase in CD34+ proliferation rates in samples frozen in hWJSC-CM and cultured in hWJSC-CM after thawing were 2.28± 0.17 [Fig. 2(b)].

CD34+ Cell Analysis

1. Cell pellets from Experimental and Control arms were blocked with 10 % NGS to prevent non-specific binding.
2. The cells were then incubated with primary anti-human CD34 antibody for 30 min followed by secondary anti-mouse IgG(H+L) antibody in the dark for 30 min.
3. The cells were washed with PBS (-), re-suspended in 10 % NGS and filtered using a 40 μM nylon strainer to remove cell clumps and analyzed with CyAn™ ADP Analyzer.
4. Greater percentages of CD34+ cells were observed in hWJSC-CM immediately after thawing compared to controls (102±1.17 %) [Fig. 2(c)].
5. Significantly greater percentages of CD34+ cells were observed when they were frozen in hWJSC-CM and cultured after thawing for 72 h in hWJSC-CM compared to controls.
6. The mean ± SEM percentage in CD34+ cells in samples frozen in hWJSC-CM and cultured in hWJSC-CM after thawing were 153.3±21.99 % [Fig. 2(d)].

CFU Assay

1. Cell pellets from Experimental and Control arms were re-suspended in 1 mL of IMDM medium supplemented with 20 % FBS, L-glutamine and antibiotic/antimycotic mixture.
2. 50 μl of the cell suspension from each group was seeded into each well of a 24-well plate containing 0.5 mL in MethoCult^R H4435 medium. The 24-wells plates were incubated at 37 °C in a 5 % CO_2 in air atmosphere for 12 days.
3. Colonies (CFU) that were formed after 12 days were classified based on morphology as described by the Atlas of Hematopoietic Colonies from Cord Blood.
4. Cells from all groups displayed typical GEMM CFU morphology as described in the literature [Fig. 3(a–d)].
5. CFU colonies were quantified using the CytoSelect™ Hematopoietic Colony Forming assay.
6. Briefly, the cells were collected and resuspended in 250 μL IMDM medium supplemented with 20 % FBS, L-glutamine and antibiotic/antimycotic mixture.
7. 50 μl of 4X Lysis Buffer/CyQuant® GR dye solution (1:75 dilution) was then added to each sample, mixed, and incubated for 30 min at room temperature.

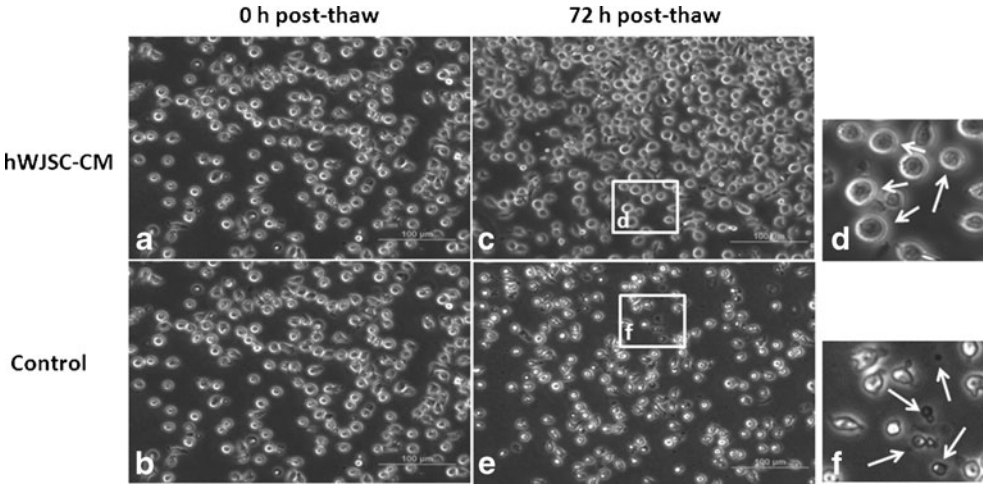

Fig. 1 **a**, **b**: Morphology of CD34+ cells frozen in hWJSC-CM, thawed and analysed immediately (0 h post-thaw). Note the thawed CD34+ cells that were frozen in hWJSC-CM having similar healthy circular morphology as controls. **c**–**f**: Morphology of CD34+ cells frozen in hWJSC-CM, thawed and then grown in hWJSC-CM for 72 h (72 h post-thaw). (**c**, **d**): Note low and high magnification of healthy CD34+ cells (*arrows*) (**e**, **f**): Controls showing greater cell death (cell debris) (*arrows*). Scale bar: 100 μm

8. 100 μl of the mixture was added to each well in the 96 well plate and readings were taken with a 485 nm/535 nm filter set using TECAN GENios.
9. Cells that were frozen in hWJSC-CM produced more CFUs compared to controls (1.07±0.02) [Fig. 3(e)].
10. More CFUs were observed when they were frozen in hWJSC-CM and cultured after thawing for 72 h in hWJSC-CM compared to controls.
11. The mean ± SEM in number of CFUs in samples frozen in hWJSC-CM and cultured in hWJSC-CM for 72 h after thawing were 1.6±0.19 [Fig. 3(f)].

Annexin V-FITC/PI Assay

1. Cell pellets from Experimental and Control arms were washed with Annexin V binding buffer (1X).

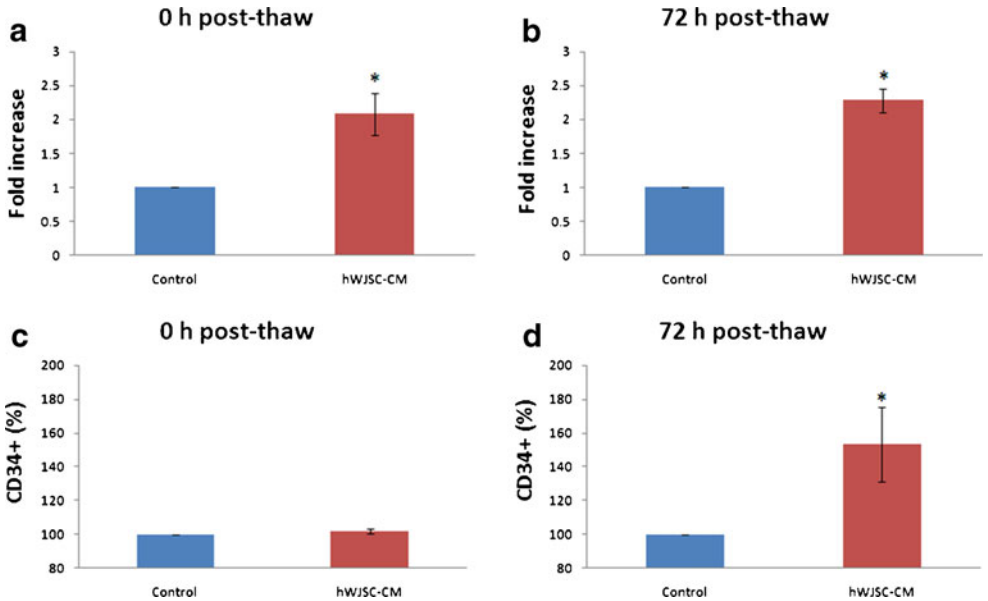

Fig. 2 Proliferation rate of CD34+ cells frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h. **a**: Note greater fold increases (normalised to respective controls) of CD34+ cells frozen in hWJSC-CM, thawed and analysed immediately (0 h post-thaw) and; **b**: Note greater fold increases of CD34+ cells after thawing and growing in hWJSC-CM for 72 h (72 h post-thaw). **c**: Note percentage increases (normalised to respective controls) of CD34+ cells frozen in hWJSC-CM, thawed and analysed immediately (0 h post-thaw); and **d** Note percentage increases of CD34+ cells after thawing and growing in hWJSC-CM for 72 h (72 h post-thaw). Values are mean ± SEM of 3 samples with 3 replicates for each sample. Asterisk (*): $p<0.05$

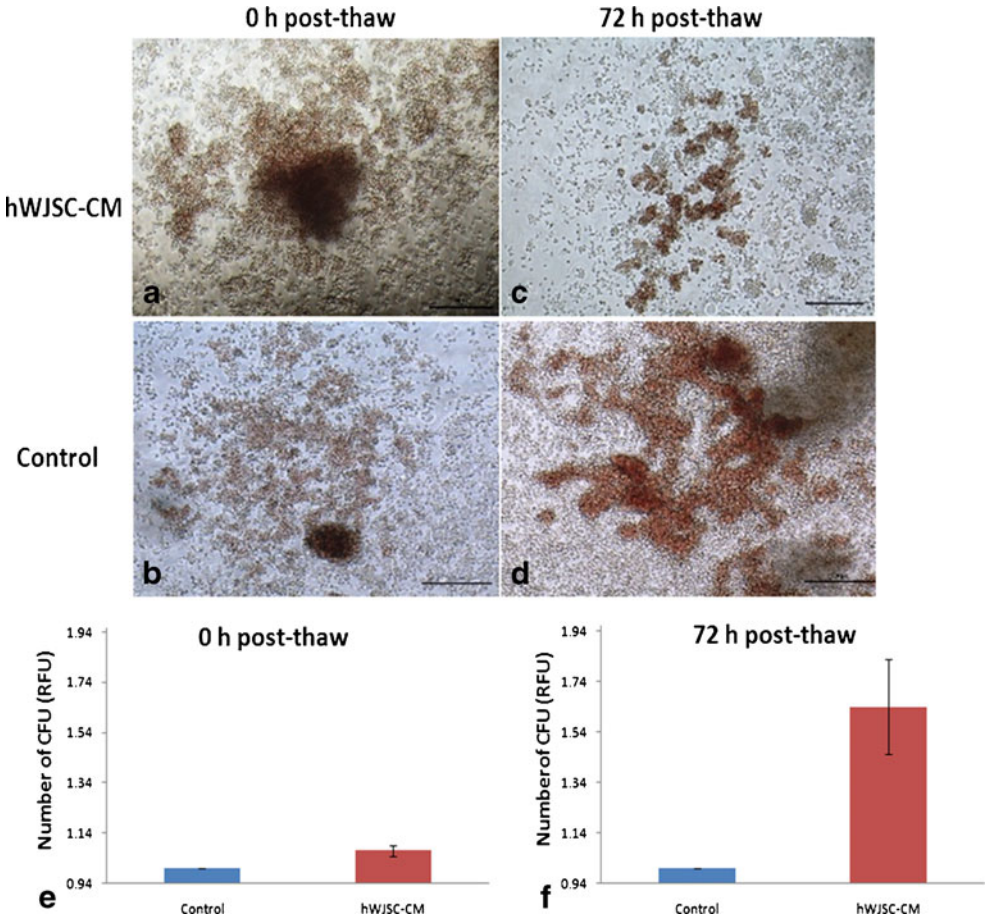

Fig. 3 CFU assay of CD34+ cells frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h. **a**: CD34+ cells frozen in hWJSC-CM, thawed and grown on methylcellulose for 12 days showed typical GEMM colonies, **b**: Parallel controls showed similar GEMM colonies. **c**: Similar GEMM colonies were observed for CD34+ cells frozen in hWJSC-CM, thawed and then grown in hWJSC-CM for 72 h. **d**: Parallel controls showed similar GEMM colonies. **e**: Greater number of colonies (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and grown on methylcellulose; and **f**: Greater number of colonies (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed, grown in hWJSC-CM for 72 h and then grown on methylcellulose. Values are mean ± SEM of 3 samples with 3 replicates for each sample

2. The cells were stained with 0.15 μg/mL Annexin V-FITC and counterstained with 1 μg/mL PI at room temperature for 15 min in the dark.
3. The cells were then filtered using a 40 μM nylon strainer to remove cell clumps and analyzed with a CyAn™ ADP Analyzer.
4. Cells frozen in hWJSC-CM had lower percentages of Annexin V+ cells compared to controls (91.06±3.63 %) [Fig. 4(a)]
5. Cells frozen in hWJSC-CM had lower percentages of PI+ cells as compared to controls (91.80±5.01 %) [Fig. 4(b)].
6. Significantly lower percentages of Annexin V+ and PI+ CD34+ cells were observed when they were thawed and cultured for 72 h in hWJSC-CM compared to controls. The mean ± SEM percentages for Annexin V+ and PI+ cells were 49.2±3.59 % and 62.0±4.30 % respectively compared to controls [Fig. 4(c) and (d)].

Live/Dead® Viability Assay

1. The staining solution was prepared by first mixing 1 μl of component A from the manufacturer's kit with 79 μl of DMSO. 1 μL of this working solution and 2 μL of component B (in the kit) were then mixed with 500 μl of PBS (-).
2. Cell pellets from Experimental and Control arms were washed with PBS (-).
3. Cells from each arm were stained with the staining solution and incubated at room temperature for 15 min in the dark.
4. Cells were then filtered using a 40 μM nylon strainer to remove cell clumps before analysis with CyAn™ ADP Analyzer.
5. Dead cells were stained red while live cells stain green. Live/dead percentages represented cell viability.
6. Greater percentages of live cells (102.3±2.32 %) were observed in samples frozen in hWJSC-CM compared to controls [Fig. 5(a)].
7. Lower percentages of dead cells (95.6±3.61 %) were observed in samples frozen in hWJSC-CM compared to controls [Fig. 5(b)].
8. Significantly higher percentages of live cells were observed when they were thawed and cultured for 72 h in hWJSC-CM compared to controls. The mean ± SEM percentage of live cells was 188.2±4.76 % compared to controls [Fig. 5(c)].
9. Significantly lower percentages of dead cells were observed when they were thawed and cultured for 72 h in hWJSC-CM compared to controls. The mean ± SEM

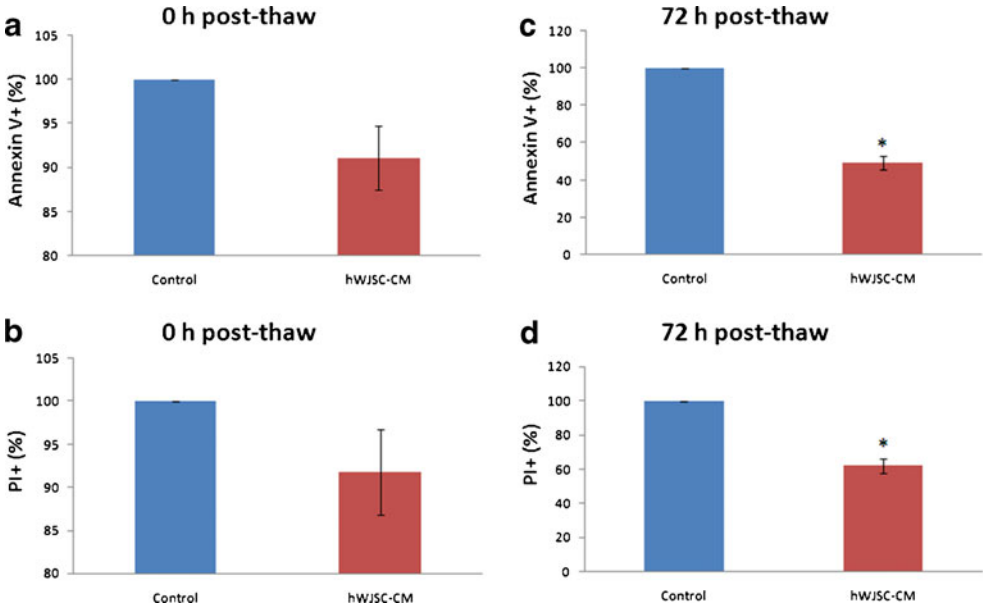

Fig. 4 Annexin V/PI assay of CD34+ cells frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h. **a**: Lower percentages of apoptotic cells (Annexin V+) (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and analysed immediately (0 h post-thaw). **b**: Lower percentages of necrotic cells (PI+) (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and analysed immediately (0 h post-thaw). **c**: Lower percentages of apoptotic cells (Annexin V+) (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h (72 h post-thaw). **d**: Lower percentages of necrotic cells (PI+) (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h (72 h post-thaw). Values are mean ± SEM of 3 samples with 3 replicates for each sample. Asterisk (*): $p<0.05$

percentage of dead cells was 56.6±5.06 % compared to controls [Fig. 5(d)].

Cell Cycle Assay

1. Cell pellets from Experimental and Control arms were fixed in 70 % ethanol overnight at −20 °C.
2. The cells were washed with PBS (-) and stained by incubating with 20 μg/mL PI and 100 μg/mL RNase A for 15 min at 37 °C in a 5 % CO_2 in air atmosphere.
3. The cells were then filtered using a 40 μM nylon strainer to remove cell clumps before analysis with a CyAn™ ADP Analyzer.
4. There were lesser percentage of cells with fragmented DNA (Sub-G1 phase) in samples frozen in hWJSC-CM compared to controls (86.1±16.26 %) [Fig. 5(e)].
5. Significantly lower percentages of cells with fragmented DNA were also observed when thawed CD34+ cells were cultured for 72 h in hWJSC-CM. The mean ± SEM percentages of cells in sub-G1 phase were 28.6± 5.74 % in hWJSC-CM compared to controls [Fig. 5(f)]. [See Notes 4 and 5].

Notes

1. Human Wharton's jelly stem cells (hWJSCs) are mesenchymal stem cells (MSCs) that can be harvested painlessly in large numbers from umbilical cords without ethical constraints and as such serve as an attractive alternative to bone marrow MSCs for cell based therapies [24, 26]. They have unique properties such as multipotency [27], hypoimmunogenicity [28] non-tumorigenicity [29], anticancer effects [20, 23] and unlike bone marrow MSCs do not form tumor-associated fibroblasts (TAFs) when cultured with cancer cells in vitro [30]. We recently reported that HSCs multiply in large numbers in vitro when in the presence of hWJSCs or hWJSC-CM and postulated that such effects may be cell-to-cell contact mediated or via molecules such as interleukins, growth factors, glycosaminoglycans and growth adhesion factors released in high concentrations into hWJSC-CM (22). The same agents in the hWJSC-CM may be playing a role in the increased freeze-thaw survival and subsequent enhanced growth of CD34+ cells observed in the present study. The cell adhesion molecules in particular may be helping to preserve the cell membrane integrity of the CD34+ cells during the freezing process while the growth factors and interleukins may enhance mitosis of the cells during the 72 h after freeze-thawing.
2. In vivo, MSCs serve as a natural scaffold within the bone marrow for the expansion of HSCs. They are analogous to a pharmacy dispensing molecules for the maturation and proliferation of CD34+ cells that eventually produce all the blood lineages into the general circulation. Such expansion of CD34+ cells is mediated

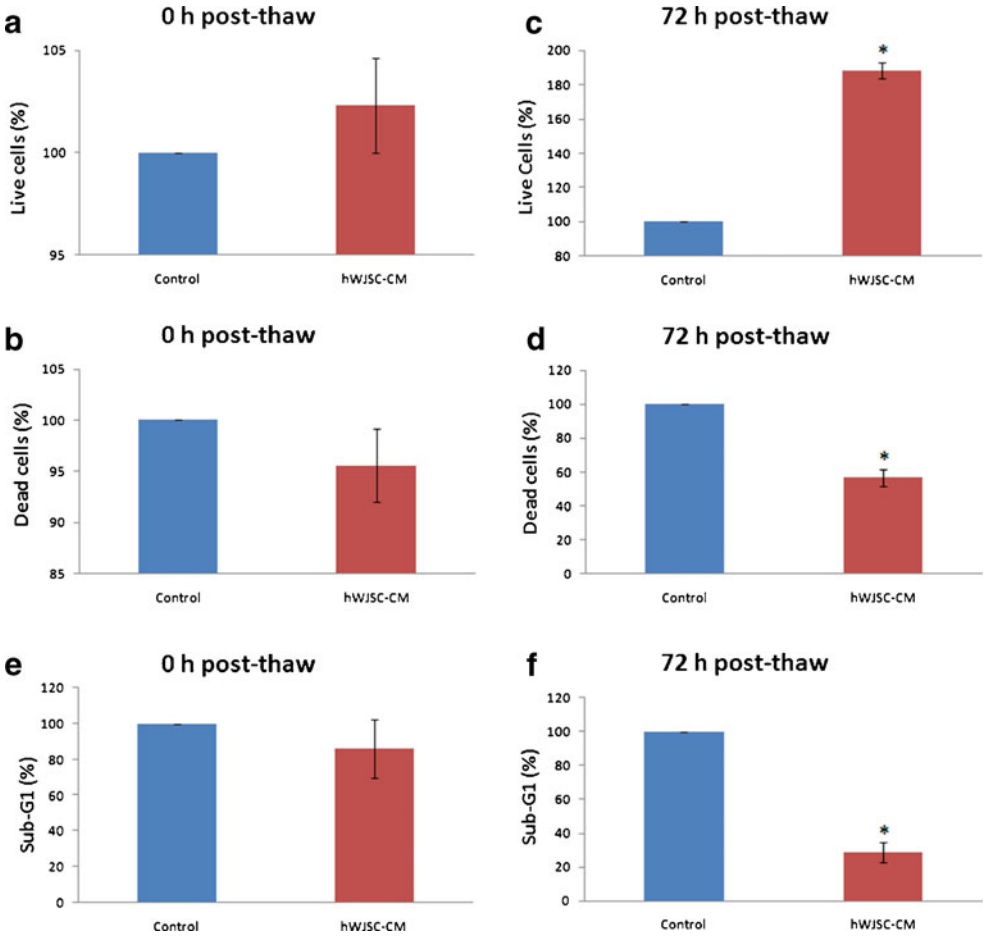

Fig. 5 Live/Dead viability and cell cycle analysis of CD34+ cells frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h. **a**: Higher percentages of live cells (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and analysed immediately (0 h post-thaw). **b**: Lower percentages of dead cells (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM thawed and analysed immediately (0 h post-thaw). **c**: Higher percentages of live cells (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h (72 h post-thaw). **d**: Lower percentages of dead cells (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h (72 h post-thaw). **e**: Lower percentages of cells with fragmented DNA (sub G1 phase) (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and analysed immediately (0 h post-thaw); and **f**: Lower percentages of cells with fragmented DNA (sub G1 phase) (normalised to controls) were observed when CD34+ cells were frozen in hWJSC-CM, thawed and grown in hWJSC-CM for 72 h (72 h post-thaw). Values are mean ± SEM of 3 samples with 3 replicates for each sample. Asterisk (*): $p<0.05$

via cell-to-cell attachment and migration into the niches of the MSC scaffold or via the molecules secreted by the MSCs. hWJSCs are also MSCs by definition and of the same embryological origin as bone marrow MSCs [31]. They are different in terms of their nature, properties and concentrations of the unique bioactive molecules they release [32]. hWJSCs appear to provide the three dimensional architecture mimicking physiological conditions ex vivo. The results of our previous studies [22] and that of the present study demonstrate that the members of the interleukin, interferon, GAGs and cell adhesion molecule families are secreted in high concentrations by hWJSCs and they singly or in combination may be the important players involved in enhanced CD34+ survival and proliferation.

3. Studies aimed at improving the cryopreservation of HSCs are focused mainly in two areas (1) refinement of the freezing media and (2) improvement of the freezing and storage procedures [33]. DMSO at a concentration of 10 % is the conventional cryoprotectant used for the freezing of HSCs in cord blood banks. However, clinically, DMSO has been known to be associated with significant side effects that include nausea, vomiting and abdominal cramps in addition to other systemic disturbances in patients receiving HSC transplantation. Therefore reducing the concentration of DMSO or buffering its effects is of vital importance in improved cryopreservation protocols for HSCs. Additionally, cryopreservation using the current conventional recipes of DMSO+ freezing media results in a significant

proportion of collected stem cells (20–30 %) becoming non-viable due to early irreversible apoptosis [34]. Various additives are being studied to improve the post-thaw recovery of CD34+ cells. Low levels of trehalose and catalase were shown to reduce post-thaw apoptosis in murine HSCs [35]. Freezing solutions supplemented with membrane stabilisers such as taurine, ascorbic acid and α-tocopheryl acetate also showed improved post-thaw recovery [36]. These reports confirm that supplements in the freezing medium can help to protect CD34+ cells from the cryoinjuries of freezing and thawing. The results of the present study using hWJSC-CM in the freezing medium showed greater post-thaw recovery of CD34+ cells compared to the use of such additives. This improvement is perhaps due to a combined effect of the interleukins, GAGs, growth factors and cell adhesion molecules present in hWJSC-CM. It was reported that the addition of certain selected interleukins to cultures of CD34+ cells induced the expansion and differentiation of these cells [37]. Hyaluronic acid which is an important member of the GAGs family was shown to stimulate the growth of CD34+ selected UCB cells into specifically differentiated mature eosinophils. This process was modulated by the CD44 receptor on the progenitor cell population [38]. Interleukin 11 stimulated the proliferation of human hematopoietic CD34+ and CD34+CD33-DR- cells and synergized with stem cell factor, interleukin-3, and granulocyte-macrophage colony-stimulating factor [39].

4. Cryopreservation involves a combination of physical and biological stresses that damages cells leading to post-thaw apoptosis [19]. CD34+ cells appear to be more vulnerable to cryoinjury compared to other somatic cells. Cryoinjury-induced apoptosis is one of the main reasons for the loss of viability after cryopreservation and such post-thaw loss of viability of CD34+ cells has always been the major factor that influences the cell numbers required for successful HSC engraftment [12–14]. Although the use of UCB-HSCs in transplantation therapy has become increasingly popular, its use has always been confined to transplantation for children because of limited viable CD34+ cell numbers [40]. Previous cryopreservation studies which examined vehicle solution, serum and protein addition, cryoprotectant and freezing curve focused only on improving the physical parameters without consideration into preventing these biological stresses [41]. Studies from adherent stem cell populations such as bone marrow MSCs and hWJSCs postulated that by inhibiting the p160-Rho-associated coiled-coil kinase signaling pathway, post-thaw apoptosis could be prevented, hence improving cell recovery and survival [18]. However, the same inhibitor fails to exhibit a similar protective effect on non-adherent UCB CD34+ cells suggesting that cell death may be occurring via a different signaling pathway [10]. The exact mechanisms as to how hWJSC-CM influences freeze-thaw survival and expansion of CD34+ cells are unclear. Preliminary results from detailed proteomic and microRNA studies on hWJSC and/or hWJSC-CM conducted by our group suggest that there is a down regulation of miR-146 which in turn results in an increase in prostaglandin E2 (PGE_2) production (Unpublished data). Recently, there appears to be increasing evidence suggesting the role of PGE_2 in the regulation of hematopoiesis and HSC engraftment [42]. It was reported that both mouse and human HSCs express PGE2 receptors and short term ex-vivo exposure to PGE_2 enhances the expansion, homing and survival of HSCs [43]. Hence the presence of PGE_2 in the hWJSC-CM may have also prevented cryoinjury-induced apoptosis by increasing intracellular *SURVIVIN* levels. Analysis of other microRNA profiles in hWJSC and hWJSC-CM will provide additional insight into the possible mechanisms that protect CD34+ cells from cryoinjury-induced apoptosis and necrosis.

5. Based on the fact that hWJSCs and hWJSC-CM support the expansion of HSCs and that hWJSC-CM also helps to enhance CD34+ thaw-survival viability and further cell proliferation after thawing, we propose that cord blood banks derive and store hWJSCs at the same time as UCB collection. An autologous supply of the same patient's hWJSCs will be an ideal adjuvant that can not only be used for cell based therapies after differentiation into a desirable lineage but can also be useful for improved freezing and expansion ex vivo. An autologous source has the added advantage of being matched to the same patient avoiding any immunorejection problems. Additionally, since hWJSCs have been reported to be hypoimmunogenic [28], allogeneic sources from other donor umbilical cords could also be stored in cord blood banks as 'off-the-shelf' cells for the same purpose. We reported that up to $4–5 \times 10^6$ live fresh hWJSCs could be obtained from 1 cm of umbilical cord and they are highly proliferative with short population doubling times [24, 26]. As such, frozen hWJSCs could be thawed and confluent monolayers established very fast for stromal support as well as for preparation of 24 h hWJSC-CM. Storage of hWJSCs or hWJSC-CM could be easily carried out in the second chamber of a dual chamber blood bag (first chamber: UCB) and the freezing protocol with/-without hWJSC-CM could be the same for the contents of both chambers. To meet this objective we also propose that hWJSC lines and hWJSC-CM be developed under current good manufacturing practice (cGMP) conditions that will be safe for patient use. hWJSC-CM is likely to face easier approval from regulatory bodies than hWJSCs

given the general concern of stem cell-associated tumorigenic risks. Thus the usage of hWJSC-CM is an appealing option as it is a safer, more defined non-cellular liquid that may have less stringent requirements from regulatory bodies for clinical application.

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Conflicts of interest All authors have no conflict of interests.

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