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Isolation, characterization, proliferation, differentiation, and freeze-thaw survival of human wharton's jelly mesenchymal stem cells from early and late passages

Rizal Rizal[1*], Fransiscus F A Kerans[1], Rio Hermantara[1], E. Henny Herningtyas[2]

[1]Biotechnology Study Program, The Graduate School of Universitas Gadjah Mada, Yogyakarta, **Indonesia**
[2]Clinical Pathology Department, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta**, Indonesia**

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***Correspondence:** rizal_biotek@yahoo.com **Accepted**: 28 Jan. 2018 **Published online:** 24 Mar. 2018

Wharton's jelly of human umbilical cord has been known to be a rich source of mesenchymal stem cells (MSCs) which have therapeutic potential for regenerative diseases. This study aimed to isolate, culture, characterize, proliferate, differentiate, and cryopreserve hWJMSCs at both early and late passages. Here hJWMSCs were isolated using explant method and propagated them up to late passage. Surface markers of hWJMSCs were analysed by flow cytometer and proliferation rate was examined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium MTS assay. Then, hWJMSCs were culture in differentiation medium and cryopreserved in different kind of freezing media. The cells at P3 and P9 were positive for CD105, CD73, CD90, and CD44 but negative for CD34, CD116, CD19, CD45, and HLA-DR. The proliferation rate of hWJMSCS at both passages increased with culture time where cell proliferation rate at P3 was higher at day 9 around 5.89±0.04 compared to P9 about 3.79±0.1 and they could be differentiated into three lineages (osteogenesis, chondrogenesis, and adipogenesis). Combination of 90% FBS and 10% DMSO showed the best cell survival rate of hWJMSCs at both passages for freeze-thawed assay. To conclude, hWJMSCs can be isolated easily using explant method, the cells retained their stem cell markers with increasing proliferation rate at both passages, showed differentiation potential and high number of live cells at cryopreservation assay.

Keywords: cryopreservation, wharton's jelly mesenchymal stem cells, explant, passages

INTRODUCTION

Mesenchymal stem cells (MSCs) have the ability for long-term self-renewal and differentiation capacity into several lineages, including osteocytes, adipocytes, chondrocytes (Baksh et al., 2007), myocytes (Quevedo et al. 2009) , cardiomyocytes (Wang et al., 2006), and neurons (Fu et al., 2006). Human mesenchymal stem cells are multipotent adult stem cells which can be derived from several sources such as adult bone marrow, fetal and adult organs, umbilical cord blood (Fong et al., 2010). The most popular source of MSCs is adult bone marrow (BM-MSCs) for research and clinical application, but isolating involves invasive procedure with the risk of infection and the number of cells decline with the age of patients as well as their biological properties are retained only for few passages (Mueller and Glowacki. 2001; Troyer and Weiss. 2008). Therefore, many studies investigated on isolation of MSCs from alternative sources such as umbilical cord (UC) tissue which can be found around perivascular regions of blood vessels, namely wharton's jelly (WJ) matrix, using different

derivation protocols (Sarugaser et al. 2005).

Human wharton's jelly mesenchymal stem cells (hWJMSCs) show both mesenchymal and embryonic stem cells marker, prolonged self-renewal, non-tumorigenic and it can be tolerated in allogeneic therapeutic use because low expression of major histocompability complex I (MHC-I) and lack of MHC-II expression (Simoes et al., 2013; Troyer and Weiss. 2008). They could be differentiated using standardized protocol into several lineages such as osteocytes, neurons, adipocytes, chondrocytes, myocardiocytes, and endothelial (Anzalone et al., 2010). Immunocytochemistry has showed that BM-MSCs and hWJMSCs share few markers such as lack of CD14, CD31, CD33, CD34, CD45, HLA-DR expression. Moreover, BM-MSCs expressed CD56, but not by hWJMSCs (Battula et al. 2009). On the other hand, at protein level, hWJMSCs expressed CD73, CD90, CD105, HLA-I as well as CD13, CD10, CD29, CD44, CD49e, and CD166 which are also markers of BM-MSCs (Christodoulou et al., 2013; Baksh et al., 2007).

hWJMSCs have greater expansion rate, higher frequency of CFU-F, shorter doubling time, more stable in terms of their surface marker expression than BM-MSCs due to its capability to maintain long telomeres until high passages (Weiss et al. 2006). Furthermore, the expression of Nanog and Oct3/4A has also played important role in maintaining long-term self-renewal and undifferentiated state (Anzalone et al., 2010). Hence, in the case of stem cell therapy, it is important to obtain the sufficient number of cells by performing long term cell culture with stable biological and morphological characteristics. Even though, hWJMSCs are found to be associated to higher proliferation rates and lower immune rejection for clinical use, an efficient cryopreservation and bio-banking are also definitely important to store hWJMSCs, which has good thaw-survival rate after freezing process. Animal serum and cryoprotectant such as DMSO has caught attention to preserve cells either in combination of different polymers or alone (Shivakumar et al., 2016). Hence, we are looking the best combination of freezing medium to cryopreserve hWJMSCs with high cell survival.

Since hWJMSCs have become more promising source of stem cell for clinical use. In this study, therefore, we evaluated the feasibility of isolating hWJMSCs using explant method, culturing them, and looking at their surface markers, proliferation rate, population doubling time (PDT), cumulative population-doubling levels (CPDLs), differentiation capabilities into three lineages, and thaw-survival efficiency from early (P3) and late (P9) passages for future regenerative medicine purposes.

MATERIALS AND METHODS

Isolation and culture of hWJMSCs

The research protocol was approved by the Institutional Review Board, Faculty of Medicine, and Universitas Gadjah Mada, Indonesia (No: KE/FK/1132/EC). Fresh umbilical cords (UC) and informed consent were collected from mothers planning on cesarian sections. All UCs were washed with sterile 0.9% sodium chloride (Otsu-NS) and sterile Phospate-buffered saline (PBS) 1x to get rid of the blood cells from UCs and processed within 24 h after delivery. Moreover, The UCs were placed into transport medium containing PBS 1x (Gibco 1740576), 1% (v/v) penicilin-streptomicin (Gibco 15140, Invitrogen, USA), 1% amphotericin-ß (v/v) (Gibco 15290026, Invitrogen Corporation, Grand Island, NY, 15290, USA) and 20 μg/ml gentamicine (Gibco 15750078) and stored at 4^{o}C for less than 24 h before tissue processing. Human wharton's jelly mesenchymal stem cells (hWJMSCs) were isolated using the explant method, according to Azandeh et al (2012). Briefly, blood vessels (artery and venous) were longitudinally dissected from the UC, separating them from the WJ tissue. The WJ tissue was then cut into 1-2 cm^3 explant fragments and washed with sterile PBS. The fragments were next plated on 60 mm tissue culture plates (MCD110090) and cultured in growth medium containing minimum essential medium-alpha (Gibco 12561056, Invitrogen, Canada) supplemented with 10% fetal bovine serum (Gibco 10270106, Invitrogen Corporation, Grand Island, NY, 14072, USA), 1% pen-strep (Gibco 15140, Invitrogen, USA), 1% amphotericin-ß (Gibco 15290026), and 20 μg/ml gentamicine (Gibco 15750078). Cultures were incubated in a humidified atmosphere with 5% CO_2 at 37 ^{o}C. The medium was replaced every 3 days.

Characterization of hWJMSCs

The expression of cell surface antigen was evaluated by flow cytometry (FACS-Calibur). Briefly, to analyze positive and negative markers of hWJMSCs, the cells were stained with appropriate positive surface antibodies as follows CD90-FITC, CD105-PerCP-Cy5, CD73-APC, CD44-PE and negative surface antibodies: CD34-PE, CD116-PE, CD19-PE, CD45-PE, HLA-DR-PE

according to manufacturer's protocol (BD stem flow Kit, 562245). Isotype controls were used as background staining. All experiments were performed in triplicate.

Proliferation assay of hWJMSCs

hWJMSCs (P3 and P9) were plated at 4 x 10^4 cells per well in six-well plates (Thermo 140675) supplemented with 2 ml of growth medium. The growth medium was replaced every 3 days. On days 3, 6, 9, 200 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium MTS assay kit (Abcam ab197010) was added into each well plate and incubated for 3h at 37 ^{0}C, 5% CO_2. The absorbance was measured using spectrophotometer (Bio-Rad microplate reader benchmark 11565) at 490 nm. The cells were trypsinized and counted on days 3, 6, and 9 for calculation of a growth kinetic curve and the population doubling (PD) between each day calculated as described previously (Ingram et al. 2004) using the equation PD = $\log_2$ (C_H/C_S), where C_H is the number of viable cells at harvest and C_S is the number of cells seeded. The population doubling time (PDT) was calculated using the time interval between cell seeding and harvest divided by the number of PDs for that passage. Cumulative population-doubling levels (CPDLs) were determined from all previous PDs at each passage. All experiments were performed in triplicate.

Osteogenic differentiation of hWJMSCs

hWJMSCs (P3 and P9) were plated at density of 2.6 x 10^4 cells /cm^2 in four-well plates (Nunc 176740, Thermo Scientific) supplemented with growth medium and incubated overnight at 37^{o}C, 5% CO_2. The next day, medium was replaced with StemPro Osteogenesis Differentiation Medium (Gibco A10072-01, Invitrogen). Medium was changed every 3 days. After 3 weeks of culture with fresh medium replacement every 3 days, the cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Osteogenesis was assessed using Alizarin Red S (Sigma A5533-25) to visualize intracellular calcium deposits.

Chondrogenic differentiation of hWJMSCs

WJMSCs (P3 and P9) were plated at density of 2.6 x 10^4 cells /cm^2 in four-well plates (Nunc 176740, Thermo Scientific) in growth medium and incubated overnight at 37^{o}C, 5% CO_2. The next day, medium was replaced with StemPro Chondrogenesis Differentiation Medium (Gibco A10071-01, Invitrogen). Medium was changed every 3 days. After 2 weeks of culture with fresh medium replacement every 3 days, the cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Chondrogenesis was confirmed by observing Alcian Blue (Sigma A5268-25) staining of extracellular matrix.

Adipogenic differentiation of hWJMSCs –

WJMSCs (P3 and P9) were plated at density of 2.6 x 10^4 cells /cm^2 in four-well plates (Nunc 176740, Thermo Scientific) in growth medium and incubated overnight at 37^{o}C, 5% CO_2. The next day, medium was replaced with StemPro Adipogenesis Differentiation Medium (Gibco A10070-01, Invitrogen). Medium was changed every 3 days. After 3 weeks of culture with fresh medium replacement every 3 days, the cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Adipogenesis was assessed using Oil Red O (Sigma O0625) to confirm intracellular lipid droplets.

hWJMSCs cryopreservation –

hWJMSCs (P3 and P9) were frozen for thaw-survival studies. For freezing, the cells were calculated around 6x10^5 cells and slowly mixed with four different freezing media containing FBS, growth medium, dimethyl sulphoxide (DMSO) and glycerol as cryprotectants. These different freezing media were FD (90% FBS and 10% DMSO), FGly (90% FBS and 10% Glycerol), FGMD (40% FBS, 50% growth medium, and 10% DMSO), and GM as negative control (100% growth medium). The cells of known counts were then placed in 1 ml cryovials (Corning 430658) and frozen in a stepwise manner started from -20 ^{o}C held for 1 hour then transferred to controlled rate cooler, Mr. Frosty, in -80 ^{o}C freezer for 24 hours and stored to a liquid nitrogen tank in three days. For thawing, the vials were removed from the liquid nitrogen tank and placed in water bath at 37 ^{o}C with gentle swirling until the cells were fully thawed, and then diluted in 2 ml of fresh growth medium. The number of living cells were then counted using trypan blue staining (Sigma T8154) for each sample to evaluate the percentages of thaw-survival efficiency.

Statistical analysis –

Data were presented as mean ± standard deviation (SD). IBM SPSS Statistics Version 22 was used to perform one-way ANOVA followed by Duncan post hoc to validate significant differences for all treatments and independent sample t-test in

order to analyze the differences between groups. A value of $P < 0.05$ was considered significant.

RESULTS

Isolation of hWJMSc with explant method

hWJMSCs were successfully isolated using explant method and cultured until passage 9. The cells grew out from the umbilical cord tissue after 5-7 days and were maintained up until two weeks (Figure 1A). The adherent cells were detached, collected and reseeded on a new T25 flask at a density 1.5 x 10^4 cells/cm^2 (Passage 0: P0). These cells displayed an epithelioid or short fibroblastic-like (star-shaped) phenotype in growth medium (Figure 1B). From the second passage onwards particularly in P3 and P9, the cells in growth medium transformed into long fibroblast spindle-shaped morphology (Figure 1C and D). There were no differences of morphology observed both at P3 and P9. The cells reached 80-90% confluency in T25 flask in about 3-4 days for each passage.

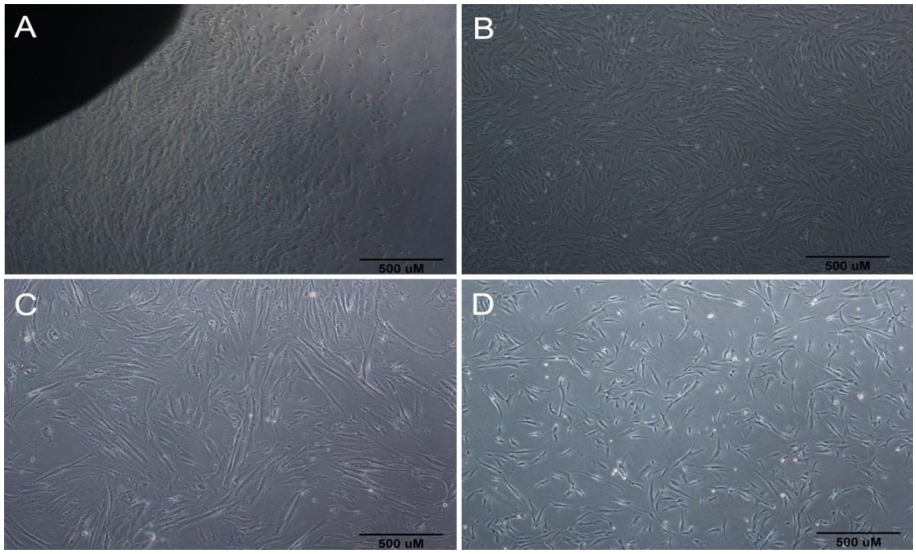


Figure 1. Morphological features of human wharton's jelly mesenchymal stem cells (hWJMSCs). (A) Migration of hWJMSCs from Wharton's jelly tissue. (B) Epitheliod-like cell morphology in P0. (C and D) Fibroblast-like cell morphology in P3 and P9 respectively. Magnification 40x. Scale Bars: 500uM.

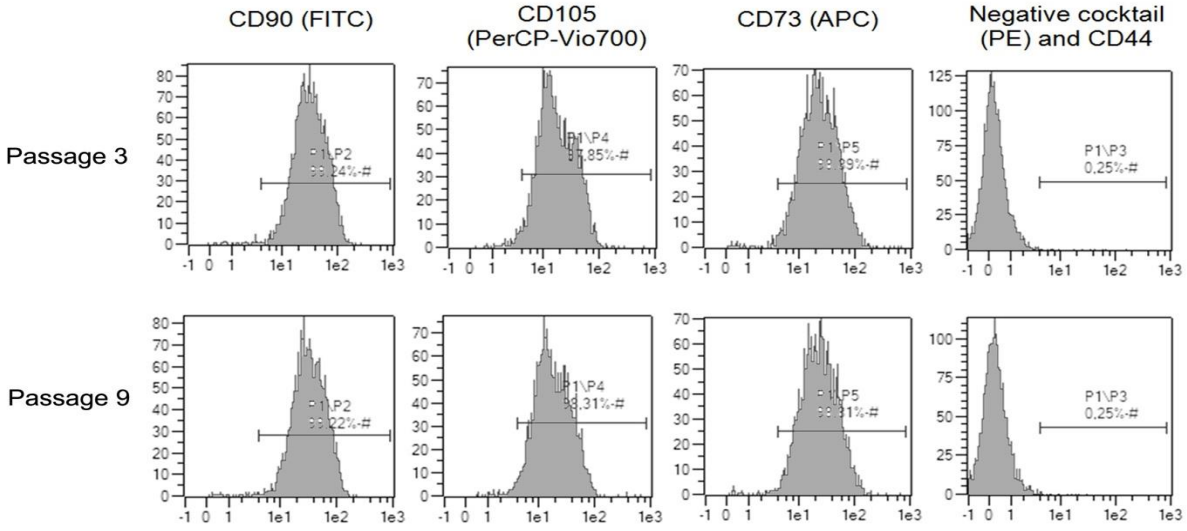


Figure. 2. Immunophenotype of human wharton's jelly mesenchymal stem cells (hWJMSCs) at both P3 and P9 detected by flow cytometric analysis.

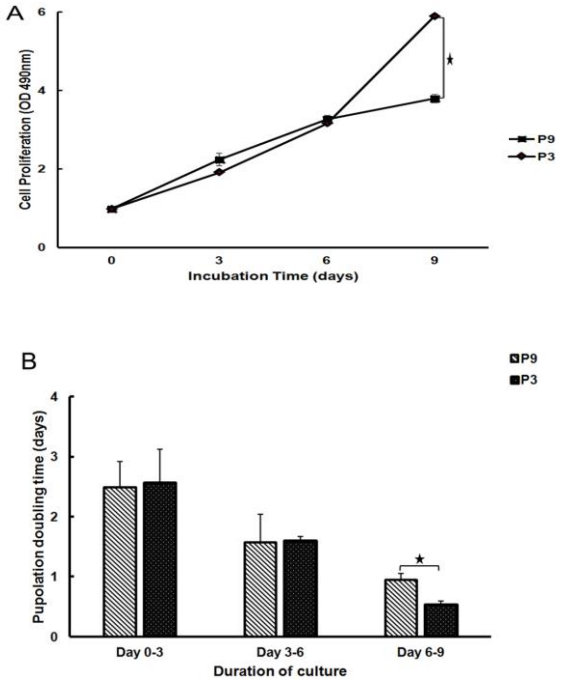
No significant difference of expression levels was identified at both passages ($P > 0.05$).

Biological properties of hWJMSCs at early and late passages

The flow cytometry analysis confirmed that isolated cells displayed cell surface markers similar to MSCs. hWJMSCs at both P3 and P9 expressed over 99% of positive surface antigenicity for CD105, CD73, CD90, and CD44. In contrast, more than 99% of the cells expressed were negative for CD34, CD116, CD19, CD45, and HLA-DR observed in both passages (Figure 2).

Cell proliferation, PDT and CPDL between early and late passages of hWJMSCs

The proliferation of hWJMSCs increased with time in growth medium. At early passage (P3), hWJMSCs grew slower than late passage (P9) at day 3 ($P < 0.05$). At day 6, however, cell proliferation was found almost similar in P3 and P9 around 3.16±0.01 and 3.27±0.09 respectively. Then, the differences in cell proliferation rate between P3 and P9 were statistically significant at day 9 about 5.89±0.04 and 3.79±0.1 respectively ($P < 0.05$) (Figure 3A). Even though, there was no difference in PDT between P3 and P9 during early phase of culture (day 0-3) and in the middle (day 3-6). At late duration of culture (day 6-9), P3 of hWJMSCs grew faster with PDT about 0.53±0.06 than P9 around 0.94±0.11 ($P < 0.05$) (Figure 3B). Moreover, there was an increase in CPDL of P3 compared to P9 ($P < 0.05$) (Figure 3C).



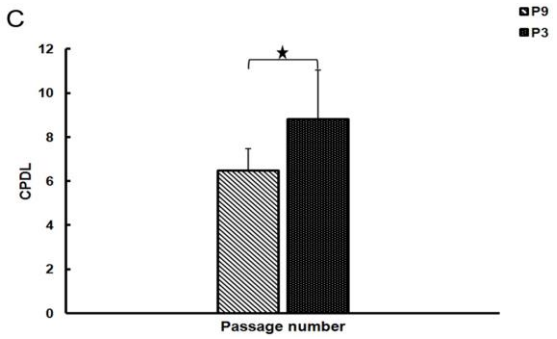


Figure. 3. Growth kinetics of human wharton's jelly mesenchymal stem cells (hWJMSCs). (A) Proliferation rate of hWJMSCs between P3 and P9 during 9 days of incubation time. (B) Population doubling time (PDT) between P3 and P9. (C) Cumulative population-doubling level (CPDL) between P3 and P9.

Bars represent the mean±SD. Significant differences were considered when *$P < 0.05$.

In vitro differentiation of hWJMSCs between early and late passages

To confirm that isolated cells exhibited the characteristic of MSCs, we differentiated them into three lineages: osteogenic, chondrogenic, and adipogenic using standard differentiation assay. For osteogenic differentiation, hWJMSCs were cultured in osteogenic differentiation medium around 21 days compared to undifferentiated hWJMSCs as negative control. Massive calcium deposition was detected by Alizarin Red S staining in osteogenic differentiated cells both at P3 and P9, as early from 14 days of culture phase. Furthermore, hWJMSCs cultured in chondrogenic medium displayed varying morphological changes from day 7 onwards with around 60% of cells changing their morphology and clustering to resemble chondrocyte structure both at P3 and P9 compared to undifferentiated cells which remained as stem cell. Extracellular matrix could be seen in chondrogenic differentiated cells after staining with Alcian Blue. Moreover, adipogenesis of hWJMSCs both at P3 and P9 were confirmed by staining the intracellular lipids with Oil Red O (Figure 4).

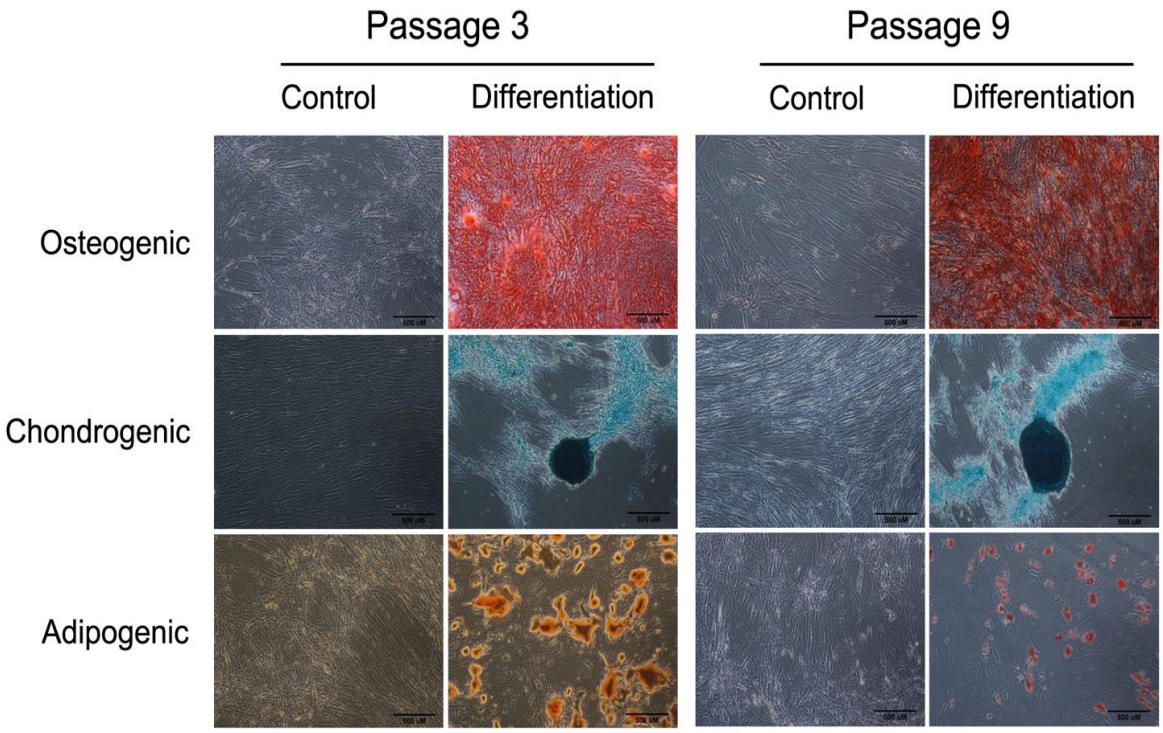
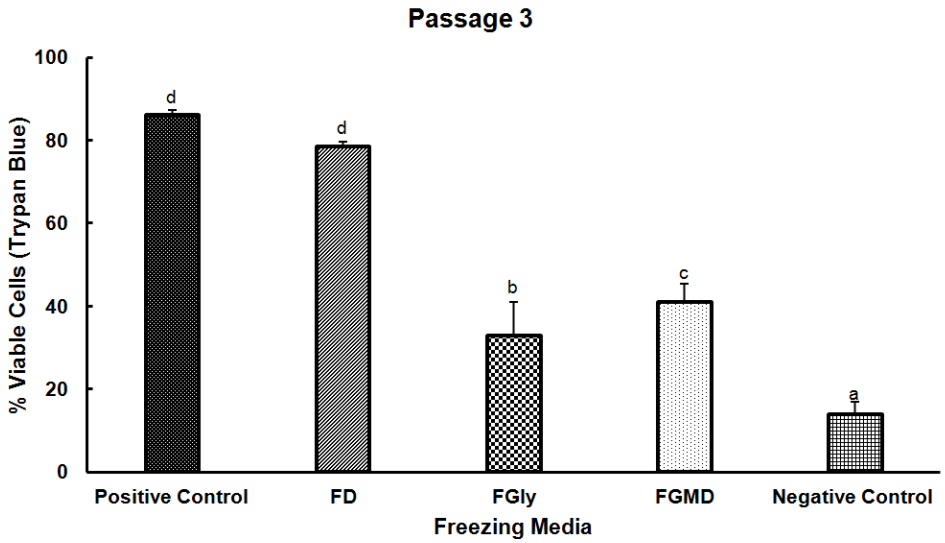


Fig.4. Osteogenic, chondrogenic, and adipogenic differentiation potentials of human Wharton's jelly mesenchymal stem cells (hWJMSCs) at P3 and P9. The cells were stained using Alizarin Red S, Alcian blue, and Oil Red O. Control was undifferentiated cells. Magnification 100x. Scale Bars: 500uM.



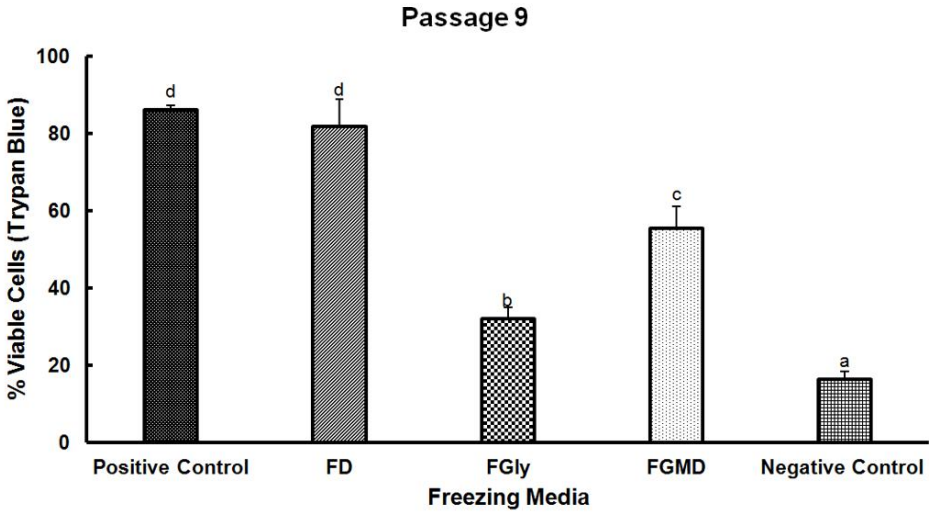


Fig. 5. Freeze-thaw viability of hWJMSCs at passage 3 (top) and passage 9 (bottom). The positive control was cell viability before freezing. The negative control was growth medium only. Abbreviations: FD: 90% FBS and 10% DMSO; FGly: 90% FBS and 10% glycerol; FGMD: 40% FBS, 50% growth medium, and 10% DMSO. Different letters $^{(a, b, c, d)}$ indicate significant differences ($P < 0.05$).

Cell survival of hWJMSCs after cryopreservation

To evaluate the number of viable cells recovered from different cryopreservation media, hWJMSCs both at P3 and P9 were stained with 0.4% trypan blue and counted using a haemocytometer. There was no significant difference of hWJMSCs viability between positive control and cryopreserved cells using combination of 90 % FBS and 10% DMSO which was the highest percentage of cell viability at both passages, followed by cryopreservation of cell using combination of 40% FBS, 50% growth medium, and 10% DMSO. Among three combinations of freezing media, combination of 90% FBS and 10% glycerol displayed the least of percentage of viable cells both at P3 and P9 around 32.89±8.14 and 31.94±2.96 respectively. On the other hand, negative control showed cell survival of P3 and P9 after freezing less than 20% compared to positive control (Figure 5).

DISCUSSION

In the present study, to examine the ability of mesenchymal stem cells (MSCs) as one of the disease treatments in this era, we isolated human wharton's jelly mesenchymal stem cells (hWJMSCs) using explant method as the efficient isolation procedure to get migratory cells moving out from wharton's jelly tissue and attaching to the plastic culture dishes during 5-7 days after explantation, which was in line with the previous study reported by (Azandeh et al., 2012). hWJMSCs were successfully isolated using enzymatic procedure (collagenase type I and hyaluronidase) but not really efficient compared to explant method (data not shown). The numbers of cells harvested from WJ tissue using explant method were higher than using enzymatic procedure which declined cell proliferation rate. This was probably due to lytic activity of enzyme affected on decreasing cell viability (Ishige et al*.,* 2009). Furthermore, the result of the present study showed when hWJMSCs were cultured in complete growth medium comprising of MEM-alpha, 10% FBS, antibiotics-antimycotics, the phenotype of hWJMSCs transformed from short fibroblastic (round shape) morphology at primary (P0 and P1) to longer-fibroblast like cells at second passage onwards. These features were similar to other findings that complex medium enhanced the hWJMSCs growth and changed their morphology compared to simple medium (Wang et al.,2004; Weiss et al., 2006).

The adherent cells obtained in this study revealed expression profile of MSCs surface markers by flow cytometric analysis. hWJMSCs both at early and late passages were positive for CD105, CD90, CD44, CD73 (MSCs markers)

while lacking the expression of CD34, CD116, CD19, CD45, and HLA-DR (hematopoietic markers). Previous study showed similar findings (Choudhery et al. 2014), validating results of this study. Lee et al*.,* (2013) reported that the expression of canine UC-MSCs surface markers (CD44, CD54, CD61, CD80, CD90 and Fik-1) was not correlated with the number of passages. However, CD105 decreased with increasing the serial passage. Moreover, Fong et al*.,* (2010) suggested that hWJMSCs retained their stem cell markers for at least 10 passages. Since these hWJMSCs at P3 and P9 could be differentiated into three different lineages (osteogenesis, chondrogenesis, and adipogenesis). It was possible that these positive CD markers expression might have contributed to multipotent capacity of hWJMSCs, that had very high percentages for both P3 and P9.

To determine the correlation between *in vitro* aging and MSCs, effect of serial passaging (passage number in culture) on proliferation and differentiation capacities of MSCs had been investigated by numerous studies. In this present study, the proliferation rate of hWJMSCs at both passages suggested similar increasing pattern from day 0 to day 9 which affected their PDT and CPDL. Previous study reported that hUC-MSCs still showed high replicative until passage 15 (Zhuang et al*.,* 2015). On the other hand, another study suggested that hUCMSCs maintained their homogenous morphology and did not lose viability with passaging even over the course of 15 passages (Shi et al*.,* 2015). hWJMSCs, in this study, did not lose their division capacity at both passage because their PDT decreased with time, suggesting that these cells retained their self-renewal capacity. This result was consistent with previous study that the mean PDT remained approximately constant until P10 (Lu et al*.,* 2006). *In vitro* aging could be reached when MSCs demonstrated maximal number of population doubling at P30 up to P40, which was related to cellular senescence where cells became enlarged and more granular and lost their proliferation potential (Banfi et al*.,* 2000).

Stem cell-based therapy has been a promising therapeutic way to regenerate defective tissue and organs. MSCs especially hWJMSCs with multipotent ability hold a great function for treating regenerative diseases. In this study, we had showed that hWJMSCs from both early and late passages still remained their ability to be differentiated into osteocytes, chondrocytes, and adipocytes assessed by specific staining. In the osteogenesis differentiation experiment, after 3 weeks of culture under osteogenic conditions, hWJMSCs displayed mineralization at both passages confirmed by high alizarin red content but lack of mineralized nodules detected in osteogenic differentiated cells. This study showed serial passaging did not change property of hWJMSCs osteogenic potential. Elkhenany et al*.* (2016) reported that low passaged BM-MSCs have the highest ability to undergo in vitro osteogenesis because the expression of osteopontin, p38, p44/42 and BMP-7. Thus, the next study must measure calcium content in order to distinguish osteogenesis ability between early and late passages. Taken together, there was no significant difference of chondrogenesis at both passages after alcian blue staining, indicating P9 was still optimal for future therapy. Even though, previous study reported that BM-MSCs and AD-MSCs at passage 12-14 suggested delayed chondrogensis and low GAGs contents, indicating that serial passaging of cells in culture changed the chondrogenic potential of these MSCs (Lee et al*.,* 2013; Elkhenany et al., 2016). Moreover, hWJMSCs in adipogenic differentiation exhibited small and dispersed lipid droplets. The pattern of adipogenesis showed morphologically similar and no obvious significant differences at both passages, suggesting hWJMSCs still retained their adipogenic potential until P9. This result was in agreement with previous study which found that serial passaging affected more to osteogenesis and chondrogenesis than adipogenesis (Kretlow et al*.,* 2008).

Cryopreservation of hWJMSCs is the efficient way to store large number of cells by freezing in liquid nitrogen without significant changes of immunophenotype, proliferation rate, and differentiation capacity for future clinical use. The most commonly cryoprotectant used along with FBS is DMSO that can limit intracellular ice formation during cryopreservation in liquid nitrogen. In this study, hWJMSCs was frozen in different media where combination of 90% FBS and 10% DMSO to be the best freezing medium in terms of viability of frozen cells compared to positive control at early and late passages, which was in agreement with previous study (Li and Cai. 2012). Moreover, cryopreservation of WJMSCs could be performed using DMSO- and serum-free cocktail cryosolution consisting of 0.05 M glucose, 0.05 M sucrose, and 1.5 M ethylene glycol in PBS for clinical utility of MSCs, which had protected the cell against freezing injury and kept stabilization of cell membranes and proteins during freezing and

drying (Shivakumar et al*.,* 2016). Hence, we performed cryopreservation of hWJMSCs using combination of 90% FBS and 10% glycerol but it showed no significant effect on the high yield of cell recovery of both passages compared to FD (FBS and DMSO) and FGMD (FBS, growth medium, and DMSO).

CONCLUSION

In conclusion, hWJMSCs could be isolated using explant method with high number of isolated cells, which was easy, safe and non-invasive. These cells exhibited stable expression of positive and negative markers of hWJMSCs at both P3 and P9. In addition, hWJMSCs retained their proliferation and differentiation capacity up to late passage and showed high cell survival rate when they cryopreserved in 90% FBS and 10% DMSO. Additional study is required to further determine differentiation capacity of hWJMSCs quantitatively.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

RR designed and performed the experiment. RR wrote the manuscript. FK, RH, and EH reviewed the manuscript. All authors read and approved the final version

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