Cryopreservation Effect on the Expression of DNA Methylation Genes in Wharton's Jelly-Derived Mesenchymal Stem Cells

Mellinda Jenuit[1], Vun Vun Hiew[2], Haselamirrah Mohd Akhir[2], Peik Lin Teoh[2#]

1 Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, MALAYSIA
2 Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, MALAYSIA.
Corresponding author. E-Mail: peiklin@ums.edu.my; Tel: +6088-320000; Fax: +6088-320993.

ABSTRACT Mesenchymal stem cells (MSCs) derived from Wharton's jelly (WJ) has become an alternative source to obtain MSCs for clinical application. This is due to less ethical concerns and invasive procedures compared to embryonic and bone marrow stem cells. To ensure plentiful supply, cryopreservation is commonly used to preserve the cell viability. However, this technique may affect the characteristics and epigenetic regulation of these cells. In this study, we investigate how cryopreservation affects genes associated with stemness, differentiation and epigenetic control. Firstly, the cell morphology and proliferation were examined under light microscope and trypan blue exclusion, respectively. Then, gene expression study was performed using RT-PCR. WJMSCs were cultured at passage 6 and 9 and cryopreserved for 2 week and 6 months. Our results showed no much change on their fibroblast-like morphology and proliferation capacity. In contrast, genes modulated the characteristics of MSCs such as NANOG, RUNX2, OCN and DNA methylation were altered after cryopreservation. In addition to other previous studies, this study has shed light on potential implications of cryopreservation on the epigenetic regulation of WJMSCs.

KEYWORDS: Cryopreservation; Stemness; Differentiation; Mesenchymal stem cells
I Received 22 February 2019 **II Revised** 08 July 2019 **II Accepted** 14 August 2019 **II In press** 22 August 2019 **II Online** 1 October 2019 **II** © **Transactions on Science and Technology I**
Full article

INTRODUCTION

Stem cell research has been receiving much of public attention ever since human embryonic stem cells (hESCs) were first grown in the lab for medical purposes. Recently, human mesenchymal stem cells (hMSCs) have been steadily progressing as the alternatives of hESCs as they do not have ethical issues and can be isolated from various tissues in an adult (Kim & Cho, 2013).

Even though hMSCs was first isolated from bone marrow, Wharton jelly-derived stem cells (WJMSCs) are preferred nowadays (Ullah *et al.*, 2015) because they possess higher cell number and less invasive procedure compared to bone marrow mesenchymal stem cells (MSCs). WJMSCs are multipotent thus they can differentiate to various cell lineages such as adipocyte, chondrocyte, osteocyte, tenocyte, fibroblast, and cardiomyocyte (Choi *et al.*, 2017). Stemness properties of MSCs refer to their ability to self-renew and remain undifferentiated which regulated by transcription factors such as *SOX2*, *OCT4* and *NANOG* (Seo *et al.*, 2013). Upon osteogenesis, the expression of master regulator, *RUNX2* and late marker for osteocytes, *OCN* are elevated. Rapid up-regulation of *CEBPα* and *CEBPß* are also occurred during induction of adipogenesis. After binding of *CEBPß* to the regulatory elements of *PPARγ* and *CEBPα*, its expression is downregulated (Chen *et al.*, 2016).

In addition, lineage differentiation of MSCs is also under epigenetic control such as DNA methylation. DNA methylation involves the addition of a methyl group to the carbon 5 of a cytosine residue, catalysed by DNA methyltransferase. DNMT1, the major DNA methyltransferase that maintaining methylation status during replication. Inactivation of DNMT1 results in delayed development and early senescence of the MSCs. DNMT3a and DNMT3b are known to catalyse *de novo* DNA methylation and *in vitro* differentiation (Tsai *et al.*, 2012).

Cryopreservation is commonly used to retain the properties of MSCs to ensure their availability. Cryopreservation involves adding cryoprotectant such as dimethyl sulphoxide (DMSO) to cells before they are stored at temperature of -80 °C and liquid nitrogen for long term storage. However, this method has been found to affect the characteristics of MSCs in terms of their morphology, proliferation rate, differentiation potential as well as the gene expression (Hunt, 2011). Therefore, this study is to examine how cryopreservation influences these characteristics in WJMSCs through the expression of genes associated with stemness, differentiation and DNA methylation.

METHODOLOGY

Cell Culture

This research was approved by UMS Ethical Committee (JKEtika 1/15(9)). Isolation of the MSCs was carried out using enzymatic digestion method described in Salehinejad *et al*. (2012) with modifications. Excepts for those indicated otherwise reagents were from Gibco (Life Technologies). Cells were cultured in DMEM/F12 media supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotic-antimycotic, and 1% (v/v) ascorbic acid (Sigma). All cultures were maintained in humidified incubator at 37 °C and 5% CO_2, and the culture medium was changed every 3 days. Cell morphology was observed under an inverted microscope.

Cryopreservation

Cells were harvested and cryopreserved using Recovery Cell Freezing Medium containing 10% (v/v) DMSO (Gibco, Life Technologies). Then, the cells were kept at -80 °C for 2 weeks and 6 months.

Proliferation assay

Proliferation assay was performed on fresh and cryopreserved cells at passage P6 and P9. The cells were cultured on 24 well plates and counted every 2 days using trypan blue exclusion method. Briefly, suspension cells were mixed with trypan blue. After mixing, cells were viewed and counted under an inverted microscope. The calculation was carried out with the following formula.

Total cells number = Number of cell counted/ 5 x dilution factor x 10^4 cells/ml

RNA extraction and RT-PCR

RNA extraction and RT-PCR were carried out using RNeasy kit and One-step RT-PCR kit (Qiagen) according to manufacturer's protocols. PCR program involved activation of HotStart DNA Taq Polymerase at 95 °C for 15 min, followed by 30 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at temperature depending on primers and extension at 72 °C for 30 s. The primers used in this study was shown in Table 1. PCR products were run on 1.5% (w/v) agarose gel.

Table 1. Primers used for RT-PCR.

Gene	Primer Sequence (5′ to 3′)
OCT4	Forward TGAGTAGTCCCTTCGCAAGC
	Reverse TTAGCCAGGTCCGAGGATCA
SOX2	Forward TTGCTGCCTCTTTAAGACTAGGA
	Reverse CTGGGGCTCAAACTTCTCTC
NANOG	Forward ACCAGAACTGTGTTCTCTTCCACC
	Reverse CCATTGCTATTCTTCGGCCAGTTG
RUNX2	Forward GACCAGTCTTACCCCTCCTACC
	Reverse CTGCCTGGCTCTTCTTACTGAG
OCN	Forward GTGCAGAGTCCAGCAAAGGT
	Reverse TCAGCCAACTCGTCACAGTC
CEBPß	Forward TTTGTCCAAACCAACCGCAC
	Reverse GCATCAACTTCGAAACCGGC
DNMT1	Forward CGGTTCTTCCTCCTGGAGAATGTCA
	Reverse CACTGATAGCCCATGCGGACCA
DNMT3A	Forward CAATGACCTCTCCATCGTCAAC
	Reverse CATGCAGGAGGCGGTAGAA
DNMT3B	Forward CCATGAAGGTTGGCGAC A
	Reverse TGGCATCAATCATCACTGGATT
ACTB	Forward GTCATTCCAAATATGAGATGCGT
	Reverse GCTATCACCTCCCCTGTGTG

RESULT AND DISCUSSION

At P6, both fresh and cryopreserved WJMSCs exhibited fibroblast spindle-like structure while at P9, both fresh and cryopreserved WJMSCs appeared to be elongated with a combination of fibroblast-like and irregular morphology after cryopreserved. However, the cell density has reduced after cryopreserved for 2 weeks and 6 months (Fig.1). Previous studies have shown that cryopreservation did not change the morphology of MSCs (Shivakumar *et al.*, 2015; Yong *et al.*, 2015; Gurruchaga *et al.*, 2017). However, the duration of cryopreservation was not clearly stated in some publications. Based on our knowledge, the reported cryopreserved duration is only 3 months.

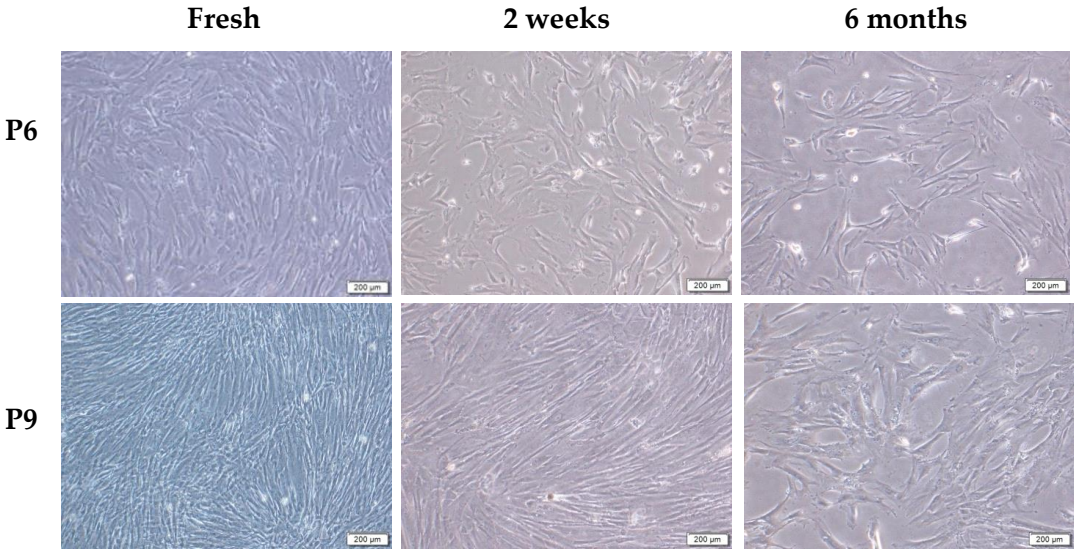

Figure 1. Morphology of fresh and cryopreserved WJMSCs. Cells are observed under 4X magnification with the scale of 200 µm.

From day 2 to 4, the number of cells at P6 and P9 for both fresh and cryopreserved were comparable (Figure 2). But at the later days, the differences in cell number in cryopreserved samples became more obvious as compared to the fresh samples. This suggests that the proliferation rate of cryopreserved WJMSCs have decreased.

In addition, genes that control stemness, differentiation and DNA methylation of WJMSCs were also altered by cryopreservation (Figure 3). The expression of all DNA methylation associated genes (*DNMT1*, *3a* and *3b*) were decreased. But the expression of *OCT4*, *NANOG* and *CEBPβ* were either maintained or increased after cryopreservation. A decrease in *OCN* expression was also noticed.

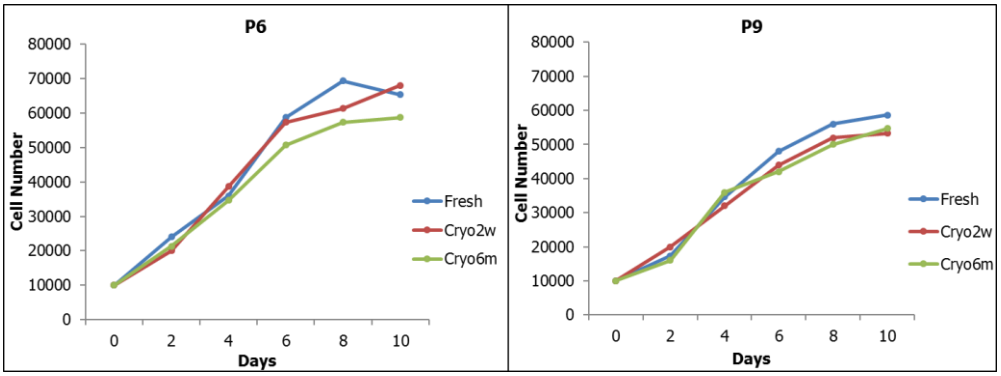

Figure 2. Cryopreservation reduces the proliferation of WJMSCs at P6 (A) and P9 (B).

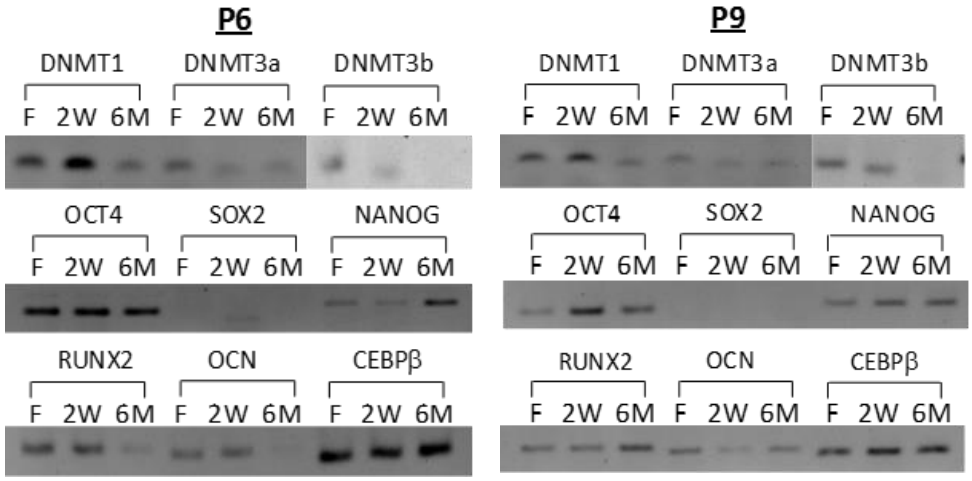

Figure 3. Cryopreservation alters the expression of genes associated with stemness, differentiation and DNA methylation at (A) P6 and (B) P9.

No obvious morphological changes were noticed although some studies report enlarged and flatten cells after cryopreservation. Knockdown of stemness genes such as *OCT4* and *SOX2* has been shown to reduce cell proliferation and multipotency of MSCs (Yoon *et al.*, 2011). In this study, cryopreserved WJMSCs retained or increased the expression of *OCT4* and *NANOG* although reduction of cell proliferation was observed. We also noticed no much changes in the expression of osteogenic gene, *RUNX2* and adipogenic gene, *CEBPβ*. Therefore, stemness properties are probably maintained after cryopreservation. Recent studies have reported that proliferative and differentiation potentials of MSCs are influenced by culture environment and oxygen level rather than stemness regulation (Pierantozzi *et al.*, 2011; Choi *et al.*, 2014; Yong *et al.*, 2015; Han *et al.*, 2012). DNA methylation also plays important roles in regulating stemness, proliferation and differentiation (Berdasco & Esteller, 2011). Decreased expression of *DNMT1*, *3a* and *3b* was observed after cryopreservation, suggesting change of epigenetic control.

CONCLUSION

These findings suggest that DNA methylation genes could be useful in determining the integrity of MSCs after cryopreservation. However, implications of long-term cryopreservation for the application MSCs for clinical setting will require further investigation.

ACKNOWLEDGEMENTS

The authors thank Ministry of Higher Education for funding this project (TRGS0003-SG-2/2014).

REFERENCES

[1] Berdasco, M. & Esteller, M. (2011). DNA methylation in stem cell renewal and multipotency. *Stem Cell Research and Therapy*, **2**(5), 42.

[2] Chen, Q., Shou, P., Zheng, C., Jiang, M., Cao, G., Yang, Q., Cao, J., Xie, N., Velletri, T., Zhang, X., Xu, C., Zhang, L., Yang, H., Hou, J., Wang, Y. & Shi, Y. (2016). Fate decision of mesenchymal stem cells: Adipocytes or osteoblasts? *Cell Death and Differentiation*, **23**, 1128-1139.

[3] Choi, J.R., Pingguan-Murphy, B., Wan Abas, W.A., Noor Azmi, M.A., Omar, S.Z., Chua, K.H. & Wan Safwani, W.K. (2014). Impact of low oxygen tension on stemness, proliferation and differentiation potential of human adipose-derived stem cells. *Biochemical and Biophysical Research Communications*, **48**, 218-224.

[4] Choi, Y.S., Park, Y-B., Ha, C-W., Kim, J.A., Heo, J-C., Han, W-J., Oh, S-Y. & Choi, S-J. (2017). Different characteristics of mesenchymal stem cells isolated from different layers of full term placenta. *PLoS One*, **12**(2), e0172642.

[5] Gurruchaga, H., del Burgo, L.S., Garate, A., Delgado, D., Sanchez, P., Orive, G., Ciriza, J., Sanchez, M. & Pedraz, J.L. (2017). Cryopreservation of human mesenchymal stem cells in an allogeneic bioscaffold based on platelet rich plasma and synovial fluid. *Scientific Reports*, **7**, 15733.

[6] Han, S., Zhao, Y., Xiao, Z., Han, J., Chen, B., Chen, L. & Dai, J. (2012). The three-dimensional collagen scaffold improves the stemness of rat bone marrow mesenchymal stem cells. *Journal of Genetics and Genomics*, **39**, 633-641.

[7] Hunt, C.J. (2011). Cryopreservation of human stem cells for clinical applications: A review. *Transfusion Medicine and Hemotherapy*, **38**, 107-123.

[8] Kim, N. & Cho, S.G. (2013). Clinical applications of mesenchymal stem cells. *Korean Journal Internal Medicine*, **28**(4), 387-402.

[9] Pierantozzi, E., Gava, B., Manini, I., Roviello, F., Marotta, G., Chiavarelli, M. & Sorrentino, V. (2011). Pluripotency regulators in human mesenchymal stem cells: expression of NANOG but not of OCT-4 and SOX-2. *Stem Cells and Development*, **20,** 915-923.

[10] Salehinejad, P., Alitheen, N.B., Nematollahi-Mahani, S.N., Ali, A.M., Omar, A.R., Janzamin, E. & Hajghani, M. (2012). Effect of culture media on expansion properties of human umbilical cord matrix-derived mesenchymal cells. *Cytotherapy*, **14**(8), 948-953.

[11] Seo, E.J., Basu-Roy, U., Gunaratne, P.H., Coarfa, C., Lim, D.S. & Basilico, C. (2013). SOX2 regulates YAP1 to maintain stemness and determine cell fate in the osteo-adipo lineage. *Cell Reports*, **3**, 2075-2087.

[12] Shivakumar, S.B., Bharti, D., Jang, S.J, Hwang, S.C., Park, J.K., Shin, J.K., Byun, J.H., Park, B.W. & Rho, G.J. (2015). Cryopreservation of human wharton's jelly-derived mesenchymal stem cells following controlled rate freezing protocol using different cryoprotectants; a comparative study. *International Journal of Stem Cells*, **8**(2), 155-169.

[13] Tsai, C.C., Su, P.F., Huang, Y.F., Yew, T.L. & Hung, S.C. (2012). Oct4 and Nanog directly regulate DNMT1 to maintain self-renewal and undifferentiated state in MSCs. *Molecular Cell*, **47**(2), 169-182.

[14] Ullah, I., Raghavendra, B.S. & Gyu, J.R. (2015). Human mesenchymal stem cells-current trends and future prospective. *Bioscience Reports*, **35**, 1-12.

[15] Yong, K.W., Pingguan-Murphy, B., Xu, F., Abas, W.A., Choi, J.R., Omar, S.Z., Azmi, M.A., Chua, K.H. & Wan Safwani, W.K. (2015). Phenotypic and functional characterization of long-term cryopreserved human adipose-derived stem cells. *Scientific Reports*, **5**, 9596.

[16] Yoon, D.S., Kim, Y.H., Jung, H.S., Paik, S. & Lee, J.W. (2011). Importance of Sox2 in maintenance of cell proliferation and multipotency of mesenchymal stem cells in low-density culture. *Cell Proliferation*, **44**, 428-440.