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Effect of Cryopreservation and cumulative population doublings on Senescence of Umbilical Cord Mesenchymal Stem Cells

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Abstract : Background: Multiple harvest explant method (MHEM) derived umbilical cord mesenchymal stem cells (UC-MSCs) was shown to undergo senescent beginning at passage-10 (P-10). However, for the same cells, there are no senescent data after cryopreservation and passage. Therefore, this study aimed to analyze the senescent profile of cryopreserved MHEM derived UC-MSCs after serial passages.
Methods: MHEM derived UC-MSCs were isolated and cultured in platelet lysate (PL) containing medium as described previously. The cells were cryopreserved at passage-1 (P-1) in 10% dimethyl sulfoxide (DMSO) and 10% PL containing alpha minimal essential medium (αMEM). Cell density at cryopreservation was 500 000 cells/mL. After one month, the cells were thawed and recultured until P-8 in six 12-well plate. At 80-90% confluent, two wells were harvested and the cells were recultured into six wells, and the four remaining wells were subjected to senescent (β-galactosidase) staining, which was done for all passages. Random photographs were taken from all stained wells, and senescent percentage was recorded.
Results: No senescent cells were observed at P-2. Senescent cells began to appear at P-3. Percentage (mean ± SD) of senescent cells from P-3 through P-8 were 0.04 ± 0.02, 1.18 ± 1.82, 0.14 ± 0.18, 0.49 ± 0.00, 0.58 ±0.91, and 0.07 ± 0.07, respectively.
Results: No senescent cells were observed at P-2. Senescent cells began to appear at P-3. Percentage (mean ± SD) of senescent cells from P-3 through P-8 were 0.04 ± 0.02, 1.18 ± 1.82, 0.14 ± 0.18, 0.49 ± 0.00, 0.58 ±0.91, and 0.07 ± 0.07, respectively.
Conclusion: No senescent cells were observed at P-2 (cumulative population doubling [CPD] > 9.38). Senescent cells began to appear at P-3 (CPD > 14.06), but in all passages until P-8 (CPD >34.34) the senescent percentage was below 5%.
Keywords: MSC, umbilical cord, cryopreservation, passage, senescence.

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Introduction

Stem cells whether in the form of a crude mixture after isolation, expanded *in vitro* or differentiated into functional cells have been shown potential to cure various kind of diseases. [1-3] Moreover, mesenchymal stem cells are increasingly used in various clinical trials and have showed promising results. [4-6] However, in vitro over expansion to provide enough number of cells, and cryopreservation to meet the need of ready to use cells might compromise cell quality. A review on the failure of phase III clinical trial, which used over expanded cryopreserved mesenchymal stem cells to cure steroid resistant graft versus host disease, supposed that the failure was due to impaired immunomodulatory functions due to cryopreservation, and senescence due to overexpansion. [7, 8] Therefore, for patients' safety and prospective comparisons between trial results, cells need to be characterized, and their function and senescent profile need to be analyzed.

Multiple harvest explant method (MHEM) derived umbilical cord mesenchymal stem cells (UC-MSCs) were shown to undergo senescent beginning at passage-10 (P-10), but until P17, the senescent percentage was below 5%. [9] Since for MHEM UCMSCs there are no senescent data after cryopreservation, this study aimed to analyze the senescent profile of cryopreserved MHEM derived UC-MSCs after serial passages. Cumulative population doublings were computed for every passages to provide comparison measures with other previous and future studies.

Materials and Methods

This was an in vitro observational study that was done June April 2014 through January 2015, in Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital - Faculty of Medicine Universitas Indonesia. Ethical approval was obtained from the Ethical Commitee of the Faculty of Medicine, Universitas Indonesia (ethical clearance No. 665/UN2.F1/ETIK/2014).

MHEM derived UC-MSCs were isolated and cultured in platelet lysate (PL) containing medium as described previously,[10]and were proven to be MSCs by their property that attached to plastics, by their surface markers, and ability to differentiate into chondrogenic, osteogenic and adipogenic lineages. The cells were cryopreserved at passage-1 (P-1) in 10% dimethyl sulfoxide (DMSO) and 10% PL containing alpha minimal essential medium (αMEM). Cell density at cryopreservation was 500,000 cells/mL, and cryopreservation procedure was done at -20^{o} C for 24 hours, and then moved into liquid N2 tank, vapor phase as described previously. [11] After one month, the cells were thawed and subjected to serial passages until P-8, and senescent analysis was done for all passages.

Thawing and serial passages

Thawing was done as quick as possible as described previously. [11] In brief, the cryotubes were immersed in a 37^{o}C water bath. After that, the cells were transferred into a complete medium, washed, and viable and total cells were counted by trypan blue exclusion method. For all passages, the cells were plated in six wells of a 12-well plate with a seeding density of 5000 viable cells/cm2. Upon 80-90% confluent, two wells were harvested and the cells were re-cultured into six wells, and the four remaining wells were subjected to senescent (β galactosidase) staining (Sigma Cat. no CS0030) as described previously.[9]

Senescent analysis

Five random photographs of all stained wells were taken using an inverted microscope connected to a digital camera, and senescent percentage from each well was recorded. As the total number of cells at 80-90% confluence was difficult to count, only senescent cells were counted, and total number of cells was interpolated using the area of photograph, area of the well, and mean value of total count from the two harvested wells.

Data collection and processing

Data collected were numbers of harvested cells from the two wells from all passages, and numbers of stained cells in the photographs. Observation and data collection of senescent cell number were done in clear and sharp photograph. The data were tabulated according to the passage and well number. The percentages of

senescent cells, and mean and standard deviation (SD) value of the senescent cells in all passages were computed. Further, cumulative population doublings at P-3 through P-8 were computed.

Results

Viability after thawing following cryopreservation was 80%. However, reculture after thawing showed that most cells were floating and only few cells were attached the next day, much fewer compared to passages of fresh cells after harvest. Floating cells were eliminated after medium change. Reculture after thawing took longer time to become confluent compared to passages of fresh cells. Population doubling at P-1, P-2, and P-3 was 4.11, 5.27, and 4.68 respectively

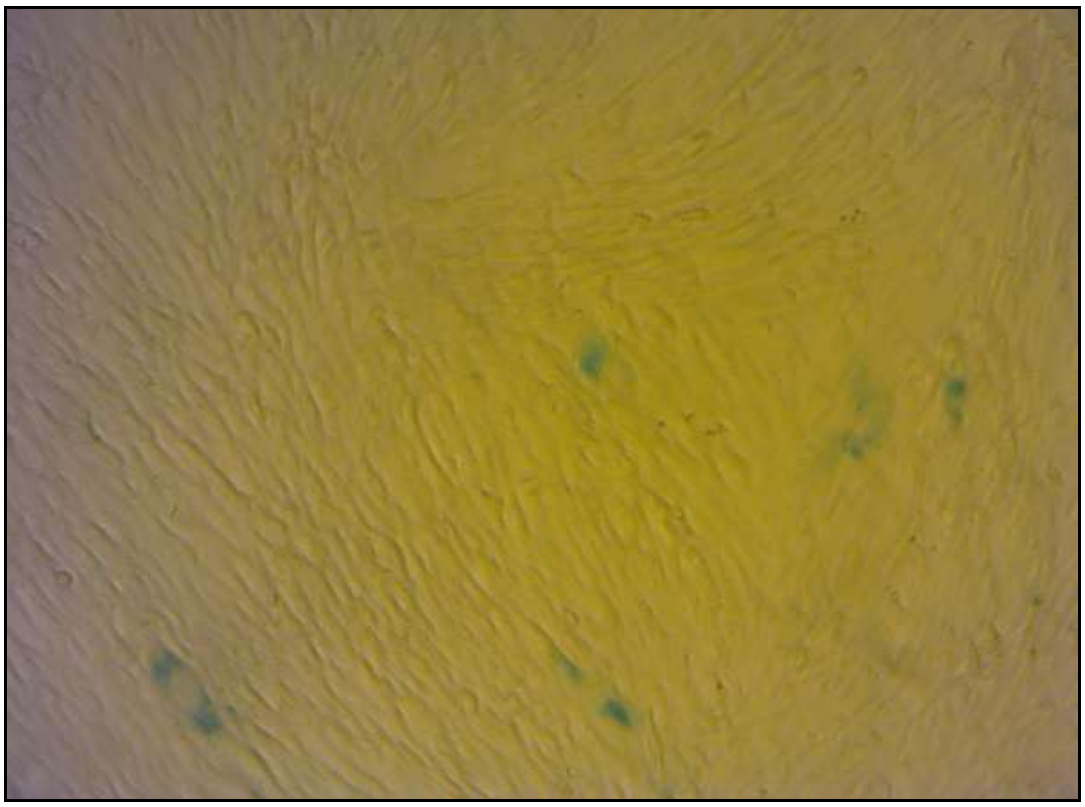

Figure 1. Microphotograph of cells in a P-3 well with non senescent and five senescent cells Senescent (β galactosidase) staining (Sigma Cat. no CS0030), blue color indicate a senescent cell, magnification 100x

No senescent cells were observed at P-2. Senescent cells began to appear at P-3 (Figure 1). Some of the wells that were intended to be stained were over confluent, and were detached after fixation. Further, some photographs were not sharp, so that they could not be analyzed. Therefore, the number of photographs that was analyzed for senescent cells numbers was incomplete. The number of photograph that were analyzed, mean and standard deviation (SD) of percentage of senescent cells, and cumulative population doubling (CPD) from P-3 through P-8 is shown in Table 1.

Table 1. Percentage of senescent cells, number of analyzed photographs, and CPDs in P-3 through P-8

	P-3	**P-4**	**P-5**	**P-6**	**P-7**	**P-8**
Mean	0.04	1.18	0.14	0.46	0.58	0.07
SD	0.02	1.82	0.18	NA	0.91	0.07
n	12	4	18	1	16	15
CPD	14.06	16.75	21.18	26.40	29.55	34.34

P-= passage-, CPD= cumulative population doublings, SD=standard deviation, NA= not applicable, n= number of analyzed photographs

Discussion

A study showed an increase in cell size with increasing passage, which was supposed to be due to senescence, [12] and another study that compared the size of senescent and non-senescent cells suggested that a non senescent cell should have a size that is less than 2602 μm^2. [9] Our previous result showed that serial passages after cryopreservation in the same method as this research caused enlargement of cell size at P-2 compared to fresh cells (mean value 2674 vs 2056 μm^2), [11] though this study showed that in P-2 there was no senescent cells. Therefore, after cryopreservation, P-2 cells in this study might begin the senescent process, but had not accumulated enough β galactosidase to be detected with β galactosidase staining, which in our study, began to detect senescence in P-3.

Our results showed a non steady increase in senescent percentage from P-3 to P-8, which showed that senescent percentages in P-5 and P-8 were lower than P-4 and P-7 respectively (Table 1). This fact might be due to the timing of senescent staining, which was the limitation of our study. Staining at 80% confluence, might give different result compared with at 90% or when the cultures were over confluence. The more confluence the culture, the more are the contacts between cells, which cause contact inhibition. Contact inhibition and serum starvation due to confluent culture may lead to quiescence, and increase in lysosomal activation and hence increased β galactosidase activity. Therefore, β galactosidase positive staining might represent senescent or quiescent cells. [13] In our study, we used β galactosidase staining at pH 6 to detect senescence associated β galactosidase activity, which can distinguish senescent from quiescent cells. Quiescent cells due to confluent culture may show weaker β galactosidase staining, which disappeared after re-culture. [14] Moreover, it has been postulated that contact inhibition suppressed senescence. [13]

To be used for patients, MSCs should be non senescent and functional. Therefore, over expansion should be avoided. Moreover, allogeneic MSCs are usually cryopreserved to match the timing of patient need and availability of cells. Cryopreservation and thawing may cause cryo injury, which have an impact on cell viability and function.

This study showed that thawing after cryopreservation caused decrease in attachment and proliferation ability, though cell viability after thawing was quite high. This fact might be due to the use of trypan blue exclusion method to assess viability directly after thawing, which was a limitation in our study. A study showed that trypan blue exclusion method only detected viable and dead cells, and not the cells that were in process of dying. [15] Further, another study showed that assessment by trypan blue exclusion method that was done directly after thawing gave higher viability compared to assessment after three days in culture, [16] when the cells in dying process were finally dead. Upon cryo injury, some cells begin cell death process due to necrosis and apoptosis that may take 6-12 hours and 12-36 hours to complete, respectively, depending on the cell type. [17] Therefore, remaining viable and functional cells that could attach and proliferate should proliferate more to attain confluence that explained the faster appearance of senescent cells, which in this study was in P-3, compared to fresh cells that began to show senescent cells at P-10. [9] Moreover, population doublings in re-culture after thawing following cryopreservation were actually more than the computed value, as we used seeding density of viable cells based on trypan blue exclusion method direct assay to compute the population doublings, while viable cell seeding density after thawing was actually much less. Therefore, cumulative population doubling was actually more than 34.34.

Expansion methods differ between labs. Therefore, comparing passage number between studies is not appropriate, and passage number should be accompanied by cumulative population doubling.

Conclusion

No senescent cells were observed at P-2 (CPD > 9.38). Senescent cells began to appear at P-3 (CPD > 14.06), but in all passages until P-8 (CPD >34.34) the senescent percentage was below 5%.

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Conflict of Interest: The authors have no conflicting financial interest.

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