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SENESCENT PROFILE OF CRYOPRESERVED ADIPOSE MESENCHYMAL STEM CELLS AFTER MULTIPLE PASSAGES

Starifulkani Arif [1], Ismail Hadisoebroto Dilogo [1,2], Jeanne Adiwinata Pawitan [2,3], Ria Anggraeni [2]
[1]Department of Orthopaedic and Traumatology, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital, Jakarta, Indonesia
[2]Stem Cell Integrated Medical Technology Unit, Cipto Mangunkusumo Hospital, Jakarta, Indonesia
[3]Department of Histology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

(Abstract) Introduction: Human adipose derived mesenchymal stem cells have generated a great deal of excitement in regenerative medicine. The need for in vitro propagation to obtain therapeutic quantities of cells imposes a risk of impaired functionality due to cellular senescence. Therefore, this study analyzed the senescent profile of previously cryopreserved human adipose mesenchymal stem cells after multiple passages. Methods: Samples of this in vitro observational analytic study that was performed from April – September 2016 were adipose mesenchymal stem cells which were cryopreserved in passage one in Stem Cell Integrated Medical Technology Unit RSCM-FKUI. After being re-cultured, the cells were cryopreserved for the second time. We compared cell size, viability, population doubling time (PDT) and senescence of first and second cryopreserved cells. Cryopreservation groups were analyzed by independent t-test and ANOVA test for each passage analysis. Results: Senescent cells appeared at passage ten in the first and passage nine in the twice cryopreserved group, respectively. In terms of once and twice cryopreservation, there were significant differences in 30% confluence cell size, PDT, percentage of senescent cells, and the size of senescent negative cell at P10 ($p<0.001$, $p<0.001$, $p< 0.001$, $p=0.032$), respectively. Meanwhile there were no differences in viability and the size of senescent positive cells at P10 ($p= 0.098$, $p= 0.212$), respectively. Conclusion: The adipose mesenchymal stem cells were senescent at the tenth passage and the ninth passage after once and twice cryopreservation, respectively. Successive cryopreservation of adipose tissue MSCs influenced cell size at senescence.

KEYWORDS: Stem Cell, Adipose Mesenchymal Stem Cell, Passage, Cryopreservation

1. INTRODUCTION

Mesenchymal stem cells (MSCs) have multipotent properties, proliferate rapidly, and have high regeneration abilities. These altogether establish tissue engineering of mesenchymal stem cells as a promising field in regeneration medicine.[1,2,3]

In recent years, the usage of adipose tissue as a source of mesenchymal stem cells is increasing. Compared to bone marrow, adipose stem cell (ASC) isolation procedures are more convenient, relatively painless, and provide more abundant cells. More over,the ability of regeneration and differentiation are the same as MSC from bone marrow.[2,4,5]

Utilization of stem cells in regenerative therapies requires quality control of the product including data of aging and cell replication (passage of cell, cell size, population doubling time, and viability). Adult stem cells can be cultured for a certain passages before they becomesenescence.[6]

In terms for clinical applications, enough MSC numbers are needed to be functional at the right time. In this case, reculturing (passage) of the MSC will provide an adequate amount of mesenchymal stem cells. Furthermore, mesenchymal stem cells need to be collected and accumulated to beused later on by a method called cryopreservation. In the process of

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cryopreservation mesenchymal stem cells will undergo cryo-injury,yet cryo-injury could be minimized by administering a variety of cryoprotectants, cooling rate, storage period and temperature. By doing so, the processmaintains the functional properties of mesenchymal stem cells including immunomodulatory properties and multilineage differentiation capabilities.[7,8,9]

Some of existing literatures described the effects of passage to MSC aging from umbilical cord.[10]However, there is still no literature that explains the aging effects of passage and cryopreservation on adipose tissue MSC.

Since the existence of excellent facilities such as Stem cells banks, allogeneic MSCs can now be provided. Yet the efficacy and safety of cryopreserved MSCs depend on the amount of cells that is cryopreserved, duration of cryopreservation and passage.

Therefore, it is necessary to do a research to evaluate the effects of passage to cryopreservedadipose MSCs. Therefore, in this study, the assessment of the effects of passage on the aging profile of cryopreserved adipose MSCs would be evaluated by cell size, population doubling time, cell viability and expression of SA-ß-Gal, which is technically relatively easy and inexpensive.

2. METHODS

This in *vitro* study was held at Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital / Faculty of Medicine Universitas Indonesia from April until September 2016. Ethical approval was issued by the ethical committee of the Faculty of Medicine, Universitas Indonesia (No 375/UN2.F1/ETIK/V/2016).

A . Materials

The study used one sample of adiposemesenchymal stem cells that were cryopreserved in passage one in Stem Cell Integrated Medical Technology Unit RSCM-FKUI. The MSCs were isolated from an adult woman adipose tissue.

B . Thawing

Cryopreserved mesenchymal stem cells in the cryotube were taken from the cryotank using insulated gloves and then thawed immediately using a water bath at 37^{o}C temperature.

C . Cell culture

Stem cell suspension that had been thawed was put in 50 mL complete medium, which contained 1 % penicillin and streptomycin, 1 % heparin, 1% amphotericin-B, 1% glutamax, and 10% platelet rich plasma in α-MEM, and was centrifuged at 2500 rpm for ten minute. The supernatant was discarded and cell pelletwas dissolved in complete medium. Cells were counted using a Neubauer Hemocytometer, and were planted into wells about 20.000 cells/cm2 for the second passage and then 5000 cells/cm2 for the third passage and subsequent repetitive passages. Then half of the harvested cells at the second passage were cryopreserved and stored again for as long as four weeks in the cryotank that contained -190^{o}C liquid nitrogen.

Each passage was done in ten wells (decuplo). Observation of the cell growth was performed every day and after two or three days the culture medium was changed. Harvest was performed when the cells were 70-80 % confluent.

D . Cryopreservation

After harvesting, the cells were stored in a cryotube in a cryo-medium that consisted of 10% of dimethylsulfoxide (DMSO) and 90% of medium (consisted of 40% of platelet rich plasma,1% penstrep, 1% heparin, 1% fungisonein alpha-MEM). A gradual cooling was done later on. The stem cells were put into a planner machine in which the temperature was gradually decreased from room temperature to -190^{o}C within 120 minutes. Stem cells were stored for one month.

E . Cell size assay

Cells were measured at 30% confluence. Cells were observed by inversion microscope and photographedat 40 times magnitude in five different visual fields. Cell areas were measured by AxioVision Rel.4.8. In each passage 50 fibroblastic cells were randomly chosen for cell measurement.

F . PDT and Viability assay

Viability and PDT were counted when the growth of the cells reached 70-80% for the third and subsequent passages. Cells were harvested using TrypLE Select (Gibco 12563-011). The harvested cells were counted by aNeubauer hemocytometer and for viability examination the tryphan blue dye exclusion method was used. PDT was calculated based on formula: t/n; n=**log (Nh)-log(Ni)/log2;** wheret= culture duration (in days), n= population doubling time, Nh= total cells that were harvested, Ni= total cells at the start. Then, some the of cells that were already counted, were sub-cultured and planted into 24 wells as much as 5000 cells/cm^2 for the fourth and subsequent passages.

G . Senescence Assay

After cell growth reached 70-80%, cells were fixed and stained with Sigma CS0030-IKT kit. Senescence cells expressed *senescence associated ß-galactosidase* (SA-ß-Gal), after 12-16 hours of incubation a blue-green color would be visible. Stained cells were observed by an inverted microscope. In each well five fields were chosen randomly and photographed at 100 times magnitude, to calculate the number of cells that was SA-ß-Gal (+).

H . Data collection and analysis

Statistical analysis was donewithSPSS v.23 that is listed on the table below

Table 1. Statistical Test

Independent Variables	Dependent Variables	Normality Test	Statistic Test
Passage	Cell Size	Saphiro-wilk	ANOVA, Tukey HSD Corr.
Passage	Viability	Saphiro-wilk	ANOVA, Tukey HSD Corr.
Passage	PDT	Saphiro-wilk	ANOVA, Tukey HSD Corr.
Passage	Senescence Cell	Saphiro-wilk	ANOVA, Tukey HSD Corr.
Passage	Senescence Cell Size	Saphiro-wilk	ANOVA, Tukey HSD Corr.
Cryopreservation	Cell Size	Saphiro-wilk	Student T test/Mann-whitney
Cryopreservation	Viability	Saphiro-wilk	Student T test/Mann-whitney
Cryopreservation	PDT	Saphiro-wilk	Student T test/Mann-whitney
Cryopreservation	Senescence Cell	Saphiro-wilk	Student T test/Mann-whitney
Cryopreservation	Senescence Cell Size	Saphiro-wilk	Student T test/Mann-whitney

3. RESULT

Below is a table of inter-cryopreservation group comparison in the cryopreservation of adipose mesenchymal stem cells. Normality test was done with the Shapiro-wilk test because the data wereless than 50. Tests between cryopreservation groups were performed using Student's t-test for normally distributed data or Mann Whitney test for data that are not normally distributed.

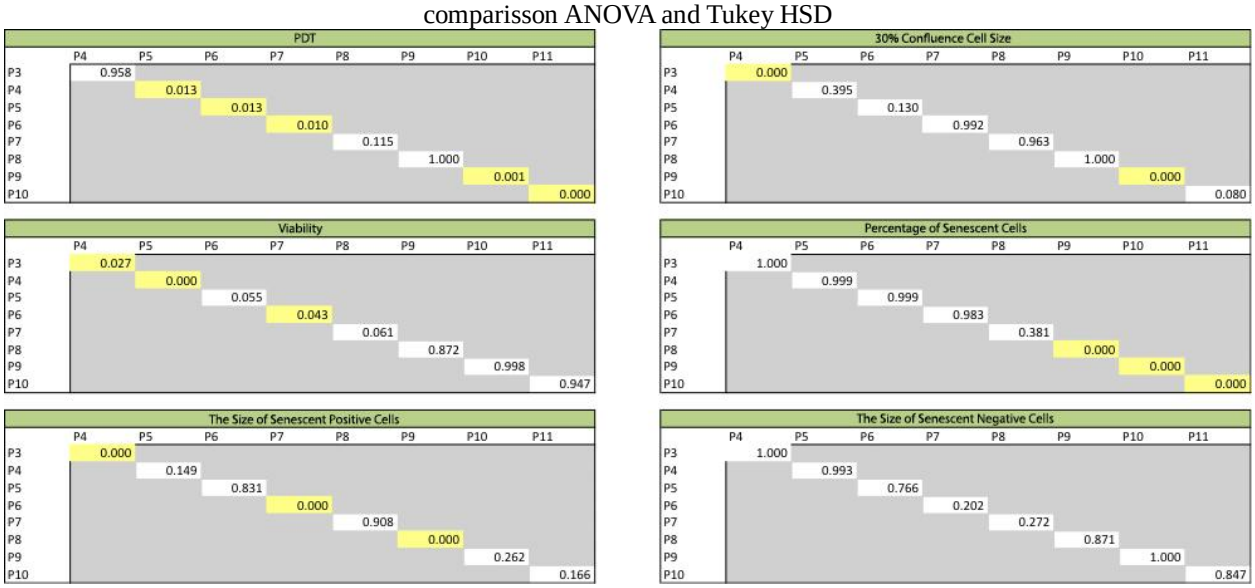
*Authors to whom correspondence should be addressed

Table 2. Difference between once and twice cryopreserved adipose mesenchymal stem cells

Variables	First cryopreservation mean(±SD)	normality	Second cryopreservation Mean (±SD)	normality	P value
Population doubling time					
P3	1.083 (0.12)	0.819			
P4	0.979 (0.04)	0.509	1.050 (0.12)	0.024	0.529**
P5	1.3 (0.14)	0.917	1.495 (0.20)	0.385	0.028*
P6	0.97 (0.10)	0.677	1.263 (0.19)	0.165	<0.001*
P7	1.31 (0.14)	0.743	1.23 (0.19)	0.013	0.130**
P8	1.55 (0.13)	0.716	1.144 (0.08)	0.612	<0.001*
P9	1.51 (0.07)	0.126	1.493 (0.069)	0.339	0.606*
P10	1.88 (0.11)	0.914	1.459 (0.18)	0.109	<0.001*
P11	3.11 (0.48)	0.013			
30% confluent cell size (µm2)					
P3	1700.95 (144.38)	0.902			
P4	2026.56 (103.92)	0.181	2067.17 (144.68)	0.683	0.114*
P5	1892.13 (224.43)	0.461	2038.29 (102.15)	0.092	0.084*
P6	1723.33 (186.16)	0.144	1725.60 (84.27)	0.682	<0.001*
P7	1666.20 (111.72)	0.958	1980.28 (139.61)	0.817	<0.001*
P8	1738.46 (78.02)	0.26	1885.82 (65.36)	0.922	<0.001*
P9	1738.46 (78.02)	0.26	1949.63 (113.44)	0.104	<0.001*
P10	2045.70 (91.40)	0.214	1763.20 (197.72)	0.033	<0.001**
P11	2226.78 (115.8)	0.465			
Viability (%)					
P3	91.76 (4.8)	0.263			
P4	97.3 (1.2)	0.042	95.36 (2.34)	0.805	0.075**
P5	88.69 (4.39)	0.618	93.98 (5.02)	0.187	0.022*
P6	93.81 (5.09)	0.387	96.35 (2.97)	0.057	<0.001*
P7	88.54 (3.64)	0.396	97.89 (2.11)	0.118	<0.001*
P8	93.59 (2.57)	0.417	95.87 (3.16)	0.126	0.093*
P9	95.68 (3.19)	0.28	93.17 (2.8)	0.47	0.053*
P10	97.17 (2.12)	0.354	95.34 (2.54)	0.507	0.098*
P11	95.17 (3.81)	0.334			
% senescence cell (%)					
P3	0				
P4	0.04 (0.03)	0.121	0.19 (0.03)	0.088	<0.001*
P5	0.17 (0.15)	0.054	0.65 (0.14)	0.459	<0.001*
P6	0.04 (0.05)	0.02	0.90 (0.30)	0.221	<0.001**
P7	0.25 (0.09)	0.874	1.04 (0.24)	0.96	<0.001*
P8	0.71 (0.21)	0.2	2.51 (0.29)	0.818	<0.001*
P9	2.24 (0.71)	0.023	4.50 (0.28)	0.086	<0.001**
P10	5.51 (0.48)	0.014	6.85 (0.26)	0.807	<0.001**
P11	10.56 (1.05)	0.268			
Senescence cell size (+)					
P3	0				
P4	2947.23 (1234.95)	0.06	6106.66 (1523.58)	0.029	<0.001**
P5	1641.76 (1503.25)	0.047	5634.73 (1214.46)	0.143	<0.001**
P6	907.6 (1209.48)	0.002	6011.28 (1759.94)	0.067	<0.001**
P7	3139.14 (715.27)	0.353	5232.50 (610.42)	0.028	<0.001**
P8	3787.54 (782.93)	0.095	5851.85 (861.93)	0.095	<0.001*
P9	6527.10 (781.71)	0.727	6546.01 (1459.66)	0.371	0.972*
P10	5352.70 (836.09)	0.984	4929.93 (882.95)	0.222	0.212*
P11	6634.75 (1578.08)	0.191			
Senescence cell size (-)					
P3	1190.58 (91.32)	0.513			
P4	1151.43 (155.11)	0.033	1532.39 (107.00)	0.495	<0.001**
P5	1218.15 (139.18)	0.811	1617.39 (179.06)	0.61	<0.001*
P6	1092.04 (95.26)	0.815	1565.77 (190.03)	0.581	<0.001*
P7	1289.19 (129.86)	0.74	1560.34 (144.22)	0.037	<0.001**
P8	1474.94 (177.19)	0.284	1664.92 (280.59)	0.378	0.09*
P9	1585.49 (206.63)	0.509	1753.54 (88.98)	0.975	0.036*
P10	1600.74 (274.58)	0.522	1376.45 (140.14)	0.765	0.032*
P11	1715.25 (176.80)	0.113			

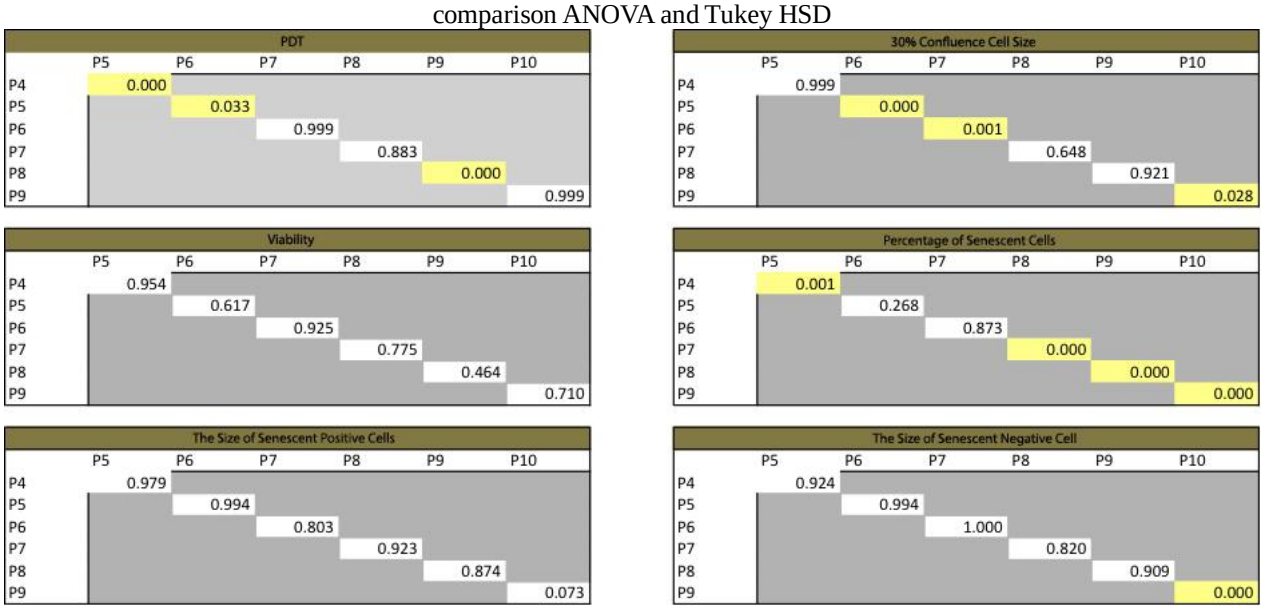
The comparison between the passagesin term of population doubling time, 30% confluence cell size, cell viability, percentage of senescence cells, as well as the senescence and non-senescence cell size in the once and twice cryopreservation groups are presented in Table 3 and Table 4. Comparison and posthoc tests were conducted using the multiple comparisons ANOVA and Tukey HSD.

Table 3. Significance test between passages of theonce cryopreserved adipose mesenchymal stem cell group using multiple comparisson ANOVA and Tukey HSD



The table above shows that there were significant differences between passages in term of senescent and non-senescent cells and population doubling time, 30% confluence cell size, and the percentage of senescence cells.

Table 4. Significance test between passages of thetwice cryopreserved adipose mesenchymal stem cell group using multiple comparison ANOVA and Tukey HSD



Based on the table above, it was concluded that there were significant differences between the passages in term of senescent and non-senescent cells, 30% confluence cell size, and the percentage of senescent and non-senescent cells.

In the once cryopreserved group, there was a significant increased in PDT at the 10^{th} passage compared to the 9^{th} passage ($p<0.05$). In the twicecryopreserved group, the PDT was increased but not as much as in the once cryopreservedgroup, and there was a significant improvement on the 9^{th} passage compared to the 8^{th} passage ($p>0.05$). Significance test between the cryopreservation groups showed no significant difference.

In terms of 30% confluence cell size, in the once cryopreserved group there was a significant increased in cell size in the 10^{th} passage compared to the 9^{th} passage ($p <0.001$). In the twice cryopreserved group, there was a non-significant increase in cell size on the 9^{th} passage compared to the 8^{th}, but the increase was significant on the 10^{th} passage ($p = 0.05$). Significance test between the cryopreservation groups showed significant improvement in the twice cryopreservation group (p

= 0.001).

In the twice cryopreservation group, viability was significantly higher compared to once cryopreservation group, but there was a significant decrease in viability in the once cryopreservation group on further passages (P9-P10; p= 0.011). In oncecryopreservation group, there was a non-significant decrease in viability at 11th compared to the 10th passage (p= 0.11). In the twice cryopreservation group, there was a non-significant increase in viability on the 10th passage (p= 1). Significance test between cryopreservation groups showed no significant differences.

At the oncecryopreservation group, there was a significant increase in the percentage of senescence cells (> 5%) started from the 10th passage (p= 0.05),while at the twice cryopreservation group, a significant increased of the percentage of senescence cells (> 5%) started from the 9th passage (p <0.001). Significance test between cryopreservation groups showed a significantly higher percentage of aging cells in the twicecryopreservation group compared to the oncecryopreservation group (p <0.001).

The size of the non-senescence cells, in the comparison between the once and twice cryopreservation groups, was found significantly increase in the P4-P7 passages (p <0.001), but no significant increase occurred in the 8th and the 9th passage. On the 10th passage, there was a significant decrease in size (p= 0.032). At the once cryopreservation group, there was an increase in cell size of aging cells between the third and last passage. Meanwhile, at the twice cryopreservation group, compared to the fourth passage, there was a slight decreasein the last passage. Both of the once and twice cryopreservation groups, there were no significant differences in changes of non-senescent cell size. Significance test between the cryopreservation groups showed no significant differences.

After being cryopreserved once, there was an increasing in cell size of the aging cells started from the 9th passage (> 4000μm). Meanwhile, at the twice cryopreservation group, increasing of the cell size of the aging cells sized > 4000 μm occurred at all passages. In the once cryopreservation group, it was shown that increased cell size was correlated to age, especially on the 11th passage (p= 0.025). Also in the twice cryopreservation group, a significant increase occurred over passages, but the cell size decreased significantly in the 10th passage (p= 0.026). Significance test showed a significant difference between two cryopreservation groups.

4. DISCUSSION

In this study, samples were divided into two groups, a once and twice cryopreservation group. First cryopreserved mesenchymal stem cells at first passage were obtained from an overweight young adult (BMI 26.3 kg.m^2, 28 years old). Choudery et al[11] and Mantovani et al.[12] stated that donor age has negative effect on senescence and proliferation ability and also differentiation of adipose mesenchymal stem cells. Meanwhile Mantovani et al.[12] stated in his study that donor age would influence significantly senescent profile of bone marrow and adipose mesenchymal stem cells. Frazier et al.[13] and Pachon-Pena et al.[14] reported the status of the body mass index in contrast to the proliferation ability, immunophenotype as well as changes in the plasticity of adipose mesenchymal stem cells.

A . Cryopreservation

The adipose mesenchymal stem cells were stored in a cryotube with 10% DMSO in 90% complete medium as cryopreservation medium. Davies et al stated that DMSO as cryo-protectant will decrease the viability of mesenchymal stem cells, because it can cause membrane cell defects, toxicity, differences in intracellular ionic concentration, and cellular hyperosmotic stress.[15] Nevertheless, Yong et al [8], on their study stated that 10% DMSO cryo-protectant gave a ratherhigher viability of mesenchymal stem cells after cryopreservation, and was in line with this study, which showed that the viability of cells after thawing was high (>91,7%). The gradual cooling technique is one of cryopreservation techniquesthat have several advantages, i.e.: high viability cell post thawing, low risk of toxicity, low contamination potency, easy procedure and require a low amount of cryo-protectant. The gradual cooling can decrease the risk of cryo-injury. James et al.[16] and Bertz et al.[17] stated that risk of cryo-injury can be decreased by the type and concentration of cryo-protectant that can penetrate and expel water from cell in order to prevent the formation of intracellular ice and cell rupture.

After adipose tissue mesenchymal stem cells were thawed quickly in 37^{o}C water bath,mesenchymal stem cells were planted in wells with ten times repetition. This repetition was useful to decrease the probability of bias, because the samples came from only one person. Yong et al.[8] stated that the quick thawing technique using water bath could be done until the whole crystal was thawed. This method can give a high recovery potency on living cells after thawing.

B . Cell Size

We found that at the beginning of the passage, adipose mesenchymal stem cells also experienced a significant increase in cell size at 30% confluence after being once cryopreserved, and a non-significant decrease was found in the next passages. Meanwhile, on the passage of cells that were already senescent (P10 at the once cryopreservation group) there was a significant increase in cell size, but instead a statistically significant decrease was found in the twice cryopreservation group (Table 2). However, the sizes were not extremely increased as in senescent cells, and might be regarded as normal size variations.

The sizes of senescent cells in once and twice cryopreservation group were extremely increased beginning at P7 and P4, respectively. Meanwhile, there were reduced sizes of senescent cells that occurred in the once and twice cryopreservation groupat P10, which were due to large cell size at earlier passages. Though cell size was reduced in P10, the size was still quite large (over 4900 μm) (Table 2). From a study conducted by Geibler et al.[18]it was found that cell size of adipose mesenchymal stem cells was 19±5 μm at P2, but at further passage (P30) dropped to 16±3 μm. Cell size would be enlarged until the cell reaches its replicative senescence, but shrink after reaching senescence. Even though the process was carried out up to the 100th passage, and the measurement was done with the flow cytometry, the samples were derived from mice.

In this study, in general, an increasing cell size after cryopreservation was linear withthe aging of cells. This is in accordance to a study conducted by Rodier et al.[19] and Hayflick et al.[6] that used human fetal diploid cells, which suggested that cell aging may cause up to 2-fold changes in cell size although the cells in that study were not cryopreserved.

C . Viability

In Adipose mesenchymal stem cells,we found that cell

viability decreaseddue to cryopreservation, where the significant decrease occurred at the passages before senescence. In the once and twice cryopreservation groups, we did not find significant differences in the cell viability between the two groups, except at P5-P7. However, viability were all above 85%, thus the differences can be regarded as variations.

D . Population Doubling Time

In adipose mesenchymal stem cells, we found a significant decreased of PDT in the twice cryopreservation group compared to once cryopreservation group. However, in the senescent adipose mesenchymal stem cells a significant increased of population doubling time was found in the once cryopreservation group. These results are in agreement with a study conducted by Gruber et al.[20] that stated that population doubling time of adipose mesenchymal stem cells would increase along passage, although the seeding density of their study was larger than in our study (50 000 cells/ well). Schellenberg et al.[21] revealed various factors may affect PDT, amongthem are: duration of culture, number of cells that were grown, harvested cell numbers, cell growth phase at harvest (log phase) and the heterogeneity of the cells that are already experiencing replicative aging. Fossett and Khan[22] mentioned in their study that the low density/ number of cells that were grown (100 cells/ cm^2) had a proliferation of 4.1 days earlier than high density (5000 cells/ cm^2). Low Population Doubling Time (PDT) in high density cells may be caused by contact inhibition, whereas when cells are planted in low density, high intake of nutrients will be provided by the medium for each cell.

E . Senescent Cells

In adipose mesenchymal stem cells, we found that the percentage of senescent cells was significantly higher in the twice cryopreservation group. This is consistent with studies of Goh et al.[23] that showed cryopreservation would reduce the efficiency of adhesion of adipose MSC (the expression of integrin α4 / CD49d), so that the proliferation of MSC will be disrupted. Therefore, the remaining cells that are able to attach and proliferate have to proliferate more to attain confluence. Moreover, the study of Choi et al[24] showed that proliferation of MSC would decline in long-term passages. Decreasing proliferation or cell division causes proliferation-able cells to proliferatemoreto achieve confluence, so that the DNA damage occurs faster, which eventually led to the Hayflick limit so the aging of cells occurs. Senescent cell size was found to be significantly increased in the twice cryopreservation group. However, Choudhery et al.[25] mentioned that the senescence of adipose mesenchymal stem cells showed the same number of cells between fresh and cryopreserved MSC.

This study need to be continuedby studies that include more samples with various characteristics (age, race, and body mass index). Cell proliferation phase at time of cryopreservation may be an important factor. Moreover, animal studies and translational researchesneed to be conducted to comparevarious passagesand senescence profiles of adipose MSCs in clinical cases like nonunion, bone defect, osteoarthritis etc.

5. CONCLUSION

Adipose mesenchymal stem cells were senescent at the tenth passage and the ninth passage after once and twice cryopreservation, respectively. Successive cryopreservation of adipose tissue MSCs influenced cell size at senescence.

6. CONFLICTS OF INTEREST

The authors declared that there are no conflicts of interest regarding the publication of this paper.

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