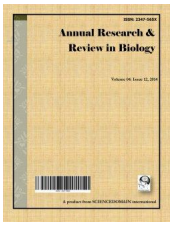

Annual Research & Review in Biology

24(1): 1-11, 2018; Article no.ARRB.39183
ISSN: 2347-565X, NLM ID: 101632869

The Passage Effect on the Senescence Profile of Cryopreserved Bone Marrow and Adipose-Derived Mesenchymal Stem Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Author HDI designed the study, own the research grant and supervised the writing of first draft of the manuscript. Author SA managed the literature searches, compiled the data and write the first draft of manuscript. Author JAP supervised the laboratory work and analysis, and revised the first draft. Author RA managed the lab work and data collection. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/39183
Editor(s):
(1) Moacir Marocolo Júnior, Professor, Physiology, Institute of Biological Sciences, Federal University of Juiz de For a, Brazil.
(2) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Alberto Olaya Vargas, Instituto Nacional de Pediatría, México.
(2) Rasul Chaudhry, Oakland University, USA.
(3) D. Atere Adedeji, Achievers University, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/23149

Original Research Article

Received 22nd November 2017
Accepted 7th February 2018
Published 12th February 2018

ABSTRACT

Background: Although bone marrow serves as the 'gold standard' MSC source, adipose tissue has become a promising alternative source. Passage and cryopreservation are two effective methods to multiply, pool, and store MSC without altering its function
Aims: To investigate the passage effects on the senescence profile of cryopreserved bone marrow and adipose-derived mesenchymal stem cells (MSCs).

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Study Design: Analytical observational study.
Place and Duration of Study: Stem Cell Medical Technology Integrated Service Unit, Faculty of Medicine, Universitas Indonesia—Cipto Mangunkusumo Hospital, Jakarta, Indonesia, during the period of April to September 2016.
Methodology: We analyzed the viability, cell size, population doubling time (PDT), percentage of senescent cells, and colony forming unit. Samples were bone marrow and adipose MSCs at passage one, which was cryopreserved for the first and second time. Numerical data were analyzed using the Student's T test and analysis of variance (ANOVA) test.
Results: Both in once and twice cryopreservation group, PDT and senescent cell percentage of bone marrow and adipose tissue MSCs differed significantly, where the PDT senescent cell percentage values of bone marrow MSCs were higher in all passages compared to adipose tissue. Regarding 30% confluence cell size and viability, significant differences between once and twice cryopreservation group varied and did not show any trend. The cell size and viability were less 2500 μm2, and more than 85%, respectively. Therefore, the difference in cell size at 30% confluence and viability might be regarded as normal variations.
Conclusion: Cryopreserved adipose-derived MSCs showed better results compared to cryopreserved bone marrow-derived MSCs in terms of population doubling time (PDT) and senescence.

Keywords: Bone marrow mesenchymal stem cell; adipose mesenchymal stem cell; passage; cryopreservation.

1. INTRODUCTION

Mesenchymal stem cells (MSCs) are very promising in the field of tissue engineering.[1-3]One source of MSCs that is most commonly used is bone marrow. However, bone marrow aspirates contain a limited number of cells, and they take a long time to reach confluence, which is approximately 3 to 4 weeks. Bone marrow aspiration may also cause trauma to the donor (donor site morbidity). Therefore, alternative resources of MSC are being sought.[4,5]

In recent years, the utilization of adipose tissue as a source of MSCs has increased. Compared to bone marrow, adipose stem cells isolation results in less pain. Moreover, the source is abundant as liposuction waste could be used. Adipose tissue also contains more MSCs compared to bone marrow, and their regeneration and differentiation ability is comparable to bone marrow MSCs.[2,4,6]

The use of stem cells in regenerative medicine needs quality control of the product, which requires viability, differentiation potential, immuno-modulating function, as well as senescent profile. Culture methods should ensure that the MSCs are non-senescent.[7]

For clinical applications, abundant MSCs should be ready in at the right time. Therefore, MSCs need to be expanded by culture. To be used at the right time, MSCs have to be stored to be used later on, cryopreservation is necessary. Cryopreservation may cause cryo-injury, yet the deleterious effect could be minimized by administrating a suitable cryo-protectant, applying slow cooling rate, and reduce storage period. Thus, by using these precautions, it is expected that cryopreserved MSCs maintain their functional properties.[8-10]

Two studies[11,12] have described the effects of the passages on the aging of fresh umbilical cord MSCs. However, there were no data that compared the combination effect of passage and cryopreservation between bone marrow and adipose MSCs. Since MSC banking has come into existence to provide cryopreserved allogenic MSCs, it is necessary to do a study to assess the effects of passage on cryopreserved bone marrow and adipose MSCs. We aim to assess the effects of passage on the aging profile of bone marrow and adipose tissue MSCs.

2. MATERIALS AND METHODS

This was an analytical observational study conducted at the Stem Cell Medical Technology Integrated Service Unit (SCMT-ISU),Faculty of Medicine, *Universitas Indonesia*—Cipto Mangunkusumo Hospital, Jakarta, Indonesia during the period of April to September 2016. This study passed the ethical review of Research Ethics Committee of the Faculty of Medicine, *Universitas Indonesia* on May 16, 2016 (No. 375 / UN2.F1 / ETHICS / V / 2016).

2.1 Samples

Samples obtained from the stem cells bank of the Stem Cell Medical Technology Integrated Service Unit (SCMT-ISU) were stored MSCs in passage one (P1). We used bone marrow and adipose tissue MSCs one cryo-tube each. Both bone marrow and adipose tissue MSCs were isolated from the same individual, a female, 28 years old with overweight nutritional status (BMI 26.3 kg/m^2).

2.2 Procedure

Both cryopreserved MSCs samples were thawed. A count of the colony-forming unit (CFU) per mililiter and subculture were performed for both samples in complete medium. Parts of the thawed MSCs were used for CFU assay, and the part was sub-cultured to P2. Once 80% confluency is achieved, the cells were harvested, and the cell number was calculated. Subsequently, the cells were cryopreserved for the second time (in 10% DMSO containing cryopreservation medium, with slow cooling rate) and subcultured. To assess their senescence and population doubling time (PDT), 5000 cells/cm2 were seeded in a 24-well plates for ten times (decuplo). For counting of the CFU per milliliter, the cells were seeded in six wells of 96-well plate at densities of 50, 25 and 12 cells per well with twice repetitions for each density. Two weeks after being cultured, the CFU were fixated and stained with Giemsa. The same step was also done in the MSCs that had been cryopreserved twice. However, in this group, 50 cells per well of 96-well plate were seeded for three times to assess their CFU. Observations of cell growth were performed every day, and the medium was changed every two-three days.

2.3 Analysis

The effects of passage on aging were evaluated by measurements of cell viability, cell size, PDT, expression of senescence-associated beta-galactosidase (SA-ß-Gal), and CFU. For both samples, the viability, cell size, population doubling time, and SA-ß-Gal staining analysis were performed for all cultures. Cell viability, PDT, and SA-ß-Gal staining were assessed when cell growth reached 70-80% starting on P3 at once, and on P4 in twice cryopreservation group. Cell measurements were performed at 30% confluence and 70-80% confluence after SA-ß-Gal staining. Cell area was measured using AxioVision program Rel.4.8 on a photograph (in μm^2). On each passage, we randomly selected 500 fibroblastic cells (spindle-shaped) for cell measurement.SA-ß-Gal staining was conducted according to manufacturer instruction of Sigma CS0030-IKT kit. SA-ß-Gal and appeared blue-green that was visible after 12-16 hours of staining. The size of random six SA-ß-Gal (+) and (-) cells was calculated using AxioVision program Rel.4.8 (in μm^2). For viability assessment, viable and dead cells were distinguished by Trypan blue dye exclusion method. The viable and dead cell numbers were counted by a Neubauer hemocytometer. The population doubling time was calculated using the following equation:

$$PDT = \frac{\log 2 \times \Delta T}{\log (NH) - \log (NI)}$$

Where ΔT is the length of culture time, NH is the number of total harvested cells and NI is the Initial seeding number.

Colony forming unit assay was performed by serial dilution and cells were cultured for 14 days, stained with Giemsa, and the numbers of formed colonies were counted.[11] Senescence assay were performed for each passage until passage ten or when there were more than 5% of senescent MSCs.[12]

2.4 Statistical Analysis

Data were analyzed using SPSS v.23. Normality test was performed using Shapiro-Wilk test. Test between cryopreservation groups was performed with Student's t-test for data with normal distribution or with Mann Whitney test for data with nonnormal distribution. A binomial sign test was done to analyze the passage effect on aging of bone marrow and adipose tissue MSCs in once and twice cryopreservation groups.

3. RESULTS AND DISCUSSION

3.1 Results

The comparison between bone marrow and adipose tissue MSCs in once and twice cryopreservation group can be seen in Table 1 and 2, respectively.

In once cryopreservation group, there were significant differences in all passages for PDT and senescent cell percentage (Table 1), where bone marrow had a higher PDT and senescent

cell percentage compared to adipose tissue MSCs. Significant differences were variable in viability, cell size at 30% confluence, and senescent cell size, where there was no significant difference in cell viability among both groups in P3 and P4, in cell size at 30% confluence in P6, and senescent cell size in P4.

In twice cryopreservation group, PDT and the percentage of cell aging were significantly different between bone marrow and adipose tissue MSCs (Table 2). In general, bone marrow aged faster than adipose tissue MSCs. Bone marrow MSC had signs of aging (> 5%) at P6, while adipose tissue MSCs at P6 showed < 5% senescent cells.

Regarding PDT, bone marrow and adipose tissue showed a significant difference both in once and twice cryopreservation group. The PDT values of bone marrow MSCs were higher in all passages compared to adipose tissue (Table 1 and 2).

Regarding 30% confluence cell size and viability, the significant difference between once and twice cryopreservation group varied and did not demonstrate any trend. Cell size and viability were all below 2500 μm2, and more than 85%, respectively. Therefore, the difference in cell size at 30% confluence and viability might be regarded as normal variations.

There was no statistically significant difference between passages of MSC in aging profile of both bone marrow and adipose tissue MSCs, both in once and twice cryopreservation group (p=0.754 and p=0.727, respectively).

Table 1. Comparison between Bone Marrow andAdipose MSCs in each passage of the once cryopreservation group

Variables	Adipose tissue		Bone marrow		*P* value
	Mean (±SD)	Normality	Mean (±SD)	Normality	
Viability (%)					
P3	91.76 (4.8)	0.263	92.64 (3.46)	0.56	0,645*
P4	97.3 (1.2)	0.042	91.04 (4.55)	0.87	<0,001**
P5	88.69 (4.39)	0.618	93.54 (1.86)	0.7	0,007*
P6	93.81 (5.09)	0.387	92.42 (5.66)	0.187	0,571*
Cell size 30% (μm2)					
P3	1700.95 (144.38)	0.902	1985.87 (95.29)	0.069	<0,001*
P4	2026.56 (103.92)	0.181	1794.35 (212.65)	0.855	0,008*
P5	1892.13 (224.43)	0.461	1600.65 (122.01)	0.787	0,003**
P6	1723.33 (186.16)	0.144	1621.07 (108.87)	0.301	0,155*
Population doubling time (days)					
P3					
P4	1.083 (0.12)	0.819	1.845 (0.163)	0.053	<0,001*
P5	0.979 (0.04)	0.509	2.336 (0.521)	0.084	<0,001*
P6	1.3 (0.14)	0.917	1.626 (0.123)	0.412	<0,001*
	0.97 (0.10)	0.677	10.359 (7.37)	0.001	<0,001**
% Senescence Cell (%)					
P3	0		0.573 (0.15)	0.347	<0,001**
P4	0.04 (0.03)	0.121	1.18 (0.29)	0.631	<0,001*
P5	0.17 (0.15)	0.054	0.229 (0.349)	0.314	<0,001*
P6	0.04 (0.05)	0.020	10.72 (1.57)	0.68	<0,001**
(+) senescence cell size (μm^2)					
P3	0		5494.40(1960.28)	<0.001	<0,001**
P4	2947.23 (1234.95)	0.060	3383.53(451.64)	0.519	0,316*
P5	1641.76 (1503.25)	0.047	5158.47(1054.99)	0.463	<0,001**
P6	907.6 (1209.48)	0.002	5353.13(774.48)	0.443	<0,001**
(-) senescence cell size (μm^2)					
P3	1190.58 (91.32)	0.513	1436.09(114.40)	0.799	<0,001*
P4	1151.43 (155.11)	0.033	1488.53(181.91)	0.228	0,001**
P5	1218.15 (139.18)	0.811	1878.53(285.08)	0.435	<0,001**
P6	1092.04 (95.26)	0.815	1778.71(334.29)	0.673	<0,001*

**Student's T test*
***Mann-Whitney U test*

Table 2. Comparison between Bone marrow and Adipose tissue MSCsin each passage of the twice cryopreservation group

Variables	Adipose Tissue		Bone marrow		*P* value
	Mean (±SD)	Normality	Mean (±SD)	Normality	
Viability (%)					
P4	95.36 (2.34)	0.805	93.51 (2.21)	0.471	0,039*
P5	93.98 (5.02)	0.187	96.64 (2.75)	0.122	0,164*
P6	96.35 (2.97)	0.057	87.44 (1.12)	0.371	<0,001*
Cell Size 30% (µm2)					
P4	2067.17 (144.68)	0.683	1965.22 (272.52)	0.423	0,314*
P5	2038.29 (102.15)	0.092	1872.26 (95.73)	0.531	0,001*
P6	1725.60 (84.27)	0.682	2005.43 (105.68)	0.025	<0,001**
Population doubling time (days)					
P4	1.050 (0.12)	0.024	2.499 (0.364)	0.445	<0,001**
P5	1.495 (0.20)	0.385	2.089 (0.145)	0.589	0,001*
P6	1.263 (0.19)	0.165	3.643 (0.114)	0.142	<0,001*
% cell senescence (%)					
P4	0.19 (0.03)	0.088	1.365 (0.171)	0.956	<0,001*
P5	0.65 (0.14)	0.459	3.10 (0.318)	0.464	<0,001*
P6	0.90 (0.30)	0.221	9.564 (0.62)	0.109	<0,001*
(+) senescene cell size (μm^2)					
P4	6106.66 (1523.58)	0.029	5742.76 (835.95)	0.335	1,000**
P5	5634.73 (1214.46)	0.143	4673.44 (890.49)	0.503	0,06*
P6	6011.28 (1759.94)	0.067	5666.19 (932.74)	0.044	0,853**
(-)cell senescence size (μm^2)					
P4	1532.39 (107.00)	0.495	1495.61 (86.54)	0.706	0,410*
P5	1617.39 (179.06)	0.61	1819.78 (976.88)	<0.001	0,481*
P6	1565.77 (190.03)	0.581	1693.06 (151.78)	1.00	0,116*

Size at 70-80% confluence of aging and non-aging MSCs in the once cryopreservation group showed variable size, and in all passages bone marrow MSCs were bigger compared to adipose MSCs; though significance was variable in aging MSCs, in nonaging MSCs, there were significant differences in all passages.

In the twice cryopreservation group, no significant differencesin size at 70-80% confluence of aging and non-aging MSCs were found between bone marrow and adipose tissue MSCs, and in all passages, the sizes of aging MSCs were all more than 2500 µm2.

Results of CFU assay of bone marrow MSCs in once cryopreservation group can be seen in Table 3. Bone marrow MSCsin the once cryopreservation group were seeded by certain densities (50, 25, and 12 cell per well) in duplicate. Mean CFU was 7.39% (Table 3). In consistencies were found in the first well where we found more colonies in lower seeding number.

Table 3. CFU of bone marrow MSCs in once cryopreservation group

Number of cell Seeding	Well 1			Well 2		
	Colony>50	Fixation day	% CFU	Colony >50	Fixation day	% CFU
50	3	14	6%	5	11	10%
25	3	11	12%	2	11	8%
12	0	14	0%	1	11	8%

Table 4. CFU of bone marrow MSCs in twice cryopreservation group

	Well 1			Well 2			Well 3		
Number of cell seeding	Colony >50	Fixation day	% CFU	Colony >50	Fixation day	% CFU	Colony >50	Fixation day	% CFU
50	3	7	6%	4	7	8%	2	7	4%

Results of CFU assay of bone marrow MSCs in twice cryopreservation group can be seen in Table 4. Colony forming unit assay was done in triplicate with a seeding number of 50 cells per well, which was determined from once cryopreservation group results. Mean CFU was 6%, and there was no colony growth in lower numbers of seeding.

Results of CFU assay of adipose tissue MSCs in once cryopreservation group can be seen in Table 5. Adipose tissue MSCsin the once cryopreservation group were seeded at 50, 25, and 12 cells per well in duplicate. For adipose MSCs at the once cryopreservation group, CFU average was 6.5%.

Results of CFU assay of adipose tissue MSCs in twice cryopreservation group can be seen in Table 6. Colony forming unit assay was done in triplicate with a seeding number of 50 cells per well. For Adipose MSC, average CFU was 17.33%.

This study used the same source of MSCs for both bone marrow and adipose-derived MSCs, which werecryopreserved in P1, and derived from a young adult individual of 28 years old. Therefore, comparison of the two sources would not be affected by individual age variation. Two studies [13,14] mentioned that donor age, which in our case was 28 years, might affect proliferation and differentiation capacity, and aging of bone marrow and adipose MSCs. However, in our comparison study, donor age would not interfere with our results, as both sources were derived from the same individual. In addition, other studies [15,16] showed that BMI status may affect proliferation and differentiation capacity of MSCs from adipose tissue. In our study, both samples were derived from an individual with an overweight status. Therefore, our results would not be affected by variation in BMI status. However, our study did not address the differentiation capacity of both MSCs, and this fact is the limitation of our study.

3.1.1 Cryopreservation and culture of bone marrow and adipose tissue MSCs

In this study bone marrow and adipose tissue MSC were stored in 10% DMSO containing cryopreservation medium. A study showed that DMSO reduced the viability of MSCs due to toxic effects, which caused damage to the cell membrane, and an increase in intracellular ion concentration, which caused hyperosmotic stress.[17] However, another study that used DMSO showed that a concentration of $\leq$ 10% would result in an utterly high viability (75%) after cryopreservation.[9] Slow cooling was used in this study; it is one of the cryopreservation techniques that has some advantages, such asthe low risk of cryo-protectant toxicity, thus reduces the risk of cryo-injury, which yields high cell viability after thawing. Several studies showed that the risk of cryo-injury could be reduced by choosing a particular type and concentration of cryo-protectants (DMS0 10-20%), which is able to penetrate and remove water from the cells thus preventing the occurrence of intracellular ice formation and rupture of cells.[18]

After bone marrow and adipose MSCs were quickly thawed in a temperature of 37^0 C, then they were washed, and recultured in decuplo. Repetitions were intended to reduce the possibility of sampling bias due to the use of only one sample each for bone marrow and adipose-derived MSCs. A study[9] mentioned that rapid thawing in a water bath melted the ice crystals. This method provides the power of self-restoration in cells after being thawed.

Table 5. CFU of adipose tissue MSCs in the oncecryopreservation group

	Well 1			Well 2		
Number of cell seeding	**Colony>50**	**Fixation day**	**% CFU**	**Colony>50**	**Fixation day**	**% CFU**
50	5	8	10%	2	8	4%
25	0	8	0%	0	8	0%
12	1	8	8%	2	8	17%

Table 6. CFU of adipose tissue MSCs in the twicecryopreservation group

	Well 1			Well 2			Well 3		
Number of cell seeding	**Colony >50**	**Fixation day**	**% CFU**	**Colony >50**	**Fixation day**	**% CFU**	**Colony> 50**	**Fixation day**	**% CFU**
50	8	7	16%	10	7	20%	8	7	16%

3.1.2 Assessment of Cell Size at 30% confluence

In this study, 30% confluence cell size, a significant difference between once and twice cryopreservation group varied and did not suggest any trend. The cell size for both groups were below 2500 μm2, and they might be considered as normal variations. These results were in line with our previous studies on bone marrow and adipose-derived MSCs, which suggested that there were significant differences of 30% confluence cell size between the senescent and non-senescent cells.[19,20]

A study [21] revealed that aging cells that were due to passages showed morphological changes from fibroblastic into large and flat heterogeneously shaped cells. However, the study was performed on P3 and P4, and there was a long duration of cryopreservation, i.e., 1.5 to 3.5 years. Moreover, the age of the donors was 27-61 years. Whereas, in this study, we compared once and twice cryopreservation in P3-P6, with a duration of one month, and a donor age of 28 years. Another study [22] did multiple cryopreservation and used permeable (0.5 M DMSO) and non-permeable (0.2 M trehalose) cryoprotectants. They showed that cell sizes tended to get bigger according to the number of cryopreservation. Though in our study cryopreservation gave a quite high number of viable cells, most cells had lost their proliferation capacity; thus remaining cells with proliferation capacity would do more cleavage to become confluent, which in turn caused aging due to the Hayflick limit was reached; thus the cells got bigger in size. Furthermore, another study[23] revealed that the size of adipose tissue MSCs in P2 was 19±5 μm, but at high passage (P30) the size was reduced to 16±3 μm. Cell size was increased until the cells reached their replicative aging and after that, the size decreased. However, samples from the study were derived from mice, cell size measurement was performed by using flow cytometry, and the passages were carried out to P100, while our study used human samples, cell size was measured using AxioVision program Rel.4.8 on a photograph, and carried out until P6.

Several studies have stated [23,24]that the size of the bone marrow MSCs that has not been cryopreserved was bigger than adipose tissue (30-120 vs. 19 μm), However, our study demonstrated that cell size at 30% confluence both in once and twice cryopreservation group, bone marrow MSCs were not consistently bigger compared to adipose MSCs. This discrepancy might be due to the method of cell size measurement in one of the studies,[23] which was done by flow-cytometry that is usually done after harvest at 70-80% confluence. At 30% confluence, MSCs might not reach their maximum size.

On the contrary, the size of MSC at 70-80% confluence of aging and non-aging MSCs in the once cryopreservation group demonstrated that bone marrow MSCs were bigger compared to adipose MSCs. Nevertheless, in the twice cryopreservation group, no significant differencesin size at 70-80% confluence of aging and non-aging MSCs were found between bone marrow and adipose tissue MSCs. This result is in line with a study [23], except in the twice cryopreservation group, where the MSCs were more involved in the aging process. This fact can be explained by several studies[7,25] that used aging human fetal diploid cells that were not cryopreserved, which found increase in cell size up to twice compared to nonaging cells.

3.1.3 Assessment of cell viability

In this study, the viability of bone marrow and adipose MSCs, and between once and twice cryopreservation group showed that significant differences were variable and did not show any trend. In addition, viabilities were all quite high (> 85%) (Table 1 and 2), thus might be regarded as normal variations. Our result in viability is supported by a study[18] which revealed that cryopreservation decreased cell viability, but cryopreservation adverse effectcould be minimized by the use of cryopreservation medium. In our study, we used DMSO containing cryopreservation medium. In assessing the viability of adipose cells, a study [18] used a combination methods of trypan blue, 3- (4,5-dimethylthiazol-2yl) - 2,5-diphenyltetrazolium bromide (MTT), and Glycerol-3-phosphate-dehydrogenase (G3DPH), as these methods were more sensitive and specific in assessing the viability of cryopreserved cells. Furthermore, another study [22] revealed that damage and cell death of cryopreserved tissue causing minimal cryo-injury still occur, even with the addition of cryo-protectants, which was supported by our study that achieved high viability.

A study [17] comparing the viability of rat adipose,bone marrow, and dental pulp MSCs found a faster decline in the viability of

cryopreserved bone marrow compared to adipose MSC, which was significant with the number of passages that might be caused by the lower amount of stem cells in bone marrow (0.001-0.1%) compared to those in adipose tissue (<10%). Besides, reduction in cell viability, in a population of bone marrow cells was associated with non-progenitor nucleated cells, which have a higher sensitivity to cryopreservation than those of adipose tissue. Moreover, bone marrow cells are more susceptible to damage that is caused by hyperosmotic cryopreservation.

A study [9] used trypan blue exclusion method, annexin V-propidium iodide, and die-life staining of cells /Calcein-A, and suggested that from several different types of cryoprotectants, ≤10% DMSO provided high MSC viability (>75%) after being cryopreserved, which corroborated the result of our study. Trypan blue concentration and incubation time during the counting process might affect the viability results in this study. Another study [26] reported that higher trypan blue concentrations (4 mg/ml) might cause toxicity that was indicated by a decrease in viability and disruption of gene expression that is associated with apoptosis and cessation of growth. Trypan blue concentration used in this study was 0.4%, which is considered non-toxic.

3.1.4 Population doubling time

In this study, PDTs of bone marrow showed a significant difference compared to those of adipose tissue, both in once and twice cryopreservation group. The PDT values of bone marrow MSCs were higher in all passages compared to adipose tissue (Table 1 and 2). Even the highest PDT value of adipose tissue MSCs was not as high as the lowest PDT value of bone marrow MSCs.

A study [27] showed that PDT of adipose MSC increased with passages, although the density of cells that were grown was larger than in this study (50.000 cells per well). Another study [28] revealed that various factors might affect PDT, among others: seeding density, culture duration, harvested cell amount, cell growth phase at harvest, and proportion of aging cells. Further, Fossett and Khan [29] showed that low seeding density (100 cells/cm^2) caused higher proliferation rate (lower PDT) compared to high seeding density (5.000 cells/cm^2). Low proliferation rate in the cells with high seeding density may be caused by contact inhibition, whereas in low seeding density more nutrients are available to every cell.

In this study, PDT obtained from bone marrow was significantly higher than adipose MSCs, in both cryopreservation groups (Tables 1 and 2). This result is supported by several studies [30,31] which showed that the PDT of bone marrow MSCs was higher than adipose MSC. This can be caused by the number of MSCs in bone marrow is less than in adipose tissue, so to achieve the same number of cells, bone marrow MSCs require a longer time, should proliferate more, and reach the Hayflick limit and age faster. Aging cells lose their proliferation ability, hence increases the overall PDT.

A study [9] reported that there were no significant differences in PDT between fresh MSCs compared to cryopreserved MSCs. However, the study used once cryopreservation only, but the duration of cryopreservation was longer. We did not compare PDT of cryopreserved versus fresh MSCs, which was one of the limitations of our study.

3.1.5 Assessment of cell aging (senescence)

In this study, the evaluation of the MSC aging was performed by computing the percentage of cell aging, and measurements of the size of aging and non aging cells. A study [32] showed that the percentage of aging cells was higher in the bone marrow compared to adipose tissue MSCs. In addition, bone marrow MSCs were larger than adipose MSCs.

In this study, there were significant differences in all passages for aging cell percentage in both once and twice cryopreservation groups. Bone marrow had a higher aging cell percentage compared to adipose tissue MSCs (Table 1 and 2). Moreover, in both once and twice cryopreservation group, bone marrow MSCs experienced aging (>5%) at P6, while at the same passage, adipose MSCs had not experienced aging (<5%). Our results were in line with a study [25] that reported increased aging percentage of bone marrow MSCs at P6. This fact be due to the less amount of bone marrow MSCs compared to those of adipose tissue. Therefore, to achieve confluence, the bone marrow MSCs should underwent mitosis more often.Thus, the DNA damage and telomere shortening that occurred during mitosis would accumulate earlier.

The size of aging and non-aging MSCs at 70-80% confluence in the once cryopreservation group suggested that bone marrow MSCs were bigger compared to adipose MSCs, in the twice cryopreservation group, no significant differences were found between bone marrow and adipose tissue MSCs, and in all passages the sizes of aging MSCs were all more than 2500 μm^2 (Table 1 and 2). This fact suggests that in the twice cryopreservation group, both bone marrow, and adipose MSCs experienced advanced aging, but in once cryopreservation group, adipose tissue MSCs experienced early aging, while bone marrow MSCs experienced advanced aging.

A study [32] has shown that cryopreservation may result in the impairment of adhesion efficiency (the expression of integrin α4/CD49d), thus the proliferation may be disrupted. Another study [13] also showed that the proliferation ability of MSC proliferation was declined in long-term passages. The decreased proliferation ability caused the need for more multiplication of proliferation-able cells to achieve confluence, which eventually led to the Hayflick limit and cell aging.

Choudhery et al. [33] stated that the peak of adipose tissue MSC aging showed the same number of aging cells between fresh and cryopreserved MSCs. However, we did not compare cryopreserved and fresh cells, which was the limitation of our study.

3.1.6 Assessment colony forming units

The bone marrow MSC CFU obtained in this study had decreased ability to form colonies in the twice cryopreservation compared to the once cryopreservation group. However, adipose MSCs showed an increase in the average CFU in the twice cryopreservation compared to the once cryopreservation group. In adipose tissue MSCs, CFU was not found until seeding density of 25 cells per well, which might be due to pipetting error. Cells that were seeded after cryopreservation might be at different state and stages of growth when cryopreserved, which contributed to inconsistencies or even the absence of colonies, even though more cells were seeded. [34] When bone marrow was compared to adipose MSC, it appeared that CFU from bone marrow was more than from adipose tissue MSCs in the once cryopreservation group, while in the twice cryopreservation group,CFU from adipose tissue MSCs was more than from bone marrow MSCs.

This study calculated the number of colonies that were formed from bone marrow and adipose tissue MSCs, and our study result in the twice cryopreservation group was in line with the result of a study [31], which reported that proliferation indicators of MSCs (one of which was CFU) from adipose tissue were better thanbone marrow. However, we did not measure the size of colonies formed, which was a limitation of this study.

4. CONCLUSION

Cryopreserved Adipose-derived MSCs showed better results compared to cryopreserved bone marrow-derived MSCs in terms of PDT and senescence.

ACKNOWLEDGEMENT

The authors would like to thank the Ministry of Research, Technology and Higher Education, for the grant: Hibah PITTA UI, Number 1753/UN2.R12/PPM.00.00/2016.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/23149