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RESEARCH ARTICLE

Passage Influence On the Aging Profile after Successive Cryopreservation of Bone Marrow Mesenchymal Stem Cells

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Abstract

Objective: Bone marrow mesenchymal stem cells are promising in cell therapy but the low frequency of this subpopulation necessitates their in vitro expansion and cryopreservation prior to clinical use. The aim of this study was to analyze the passage effect on the aging profile of previously successive cryopreserved bone marrow mesenchymal stem cells.Methods: The samples of this in-vitro observational analytic study (April - September 2016) were iliac crest bone marrow mesenchymal stem cells that were cryopreserved in passage one and a second group was cryopreserved after being subcultured. It was obtained from UPT TK Sel Punca RSCM-FKUI. Samples were analyzed for each passage in terms of cell size, viability, population doubling time (PDT), and percentage of senescent cells by ANOVA test and independent t-test for the inter-cryopreservation group.Results:The remarkable manifestation of senescence appeared at passage six in both cryopreserved groups, but tend to appear earlier in the twice cryopreservation group. The senescent positive cell size became bigger along with the increasing number of passage. There were significant differences in PDT, 30% confluent cell size, viability, in passage six between the two cryopreservation groups ($p<0.001$, $p<0.001$, $p=0.022$), respectively, but no significant difference in percentage of senescent cells ($p= 0.052$). The cells became senescent in P6 in both groups ($P=0.000$). Conclusion: The onset of senescence of bone marrow mesenchymal stem cells was in passage six, while PDT, cell size and viability differ significantly due to successive cryopreservation.

Introduction

Stem cells are cells that can regenerate by themselves and are able to differentiate into specific mature cells. A type of stem cells is mesenchymal stem cells (MSCs) which are multi-potent, and have the property of rapid proliferation, and high regeneration ability, thus making it a promising entity in the field of tissue engineering [1-3]. As previously known, MSCs are adult stem cells that can be cultured for a limited passage before they undergo senescence [4].

For clinical applications, a high amount of functional MSCs are needed. In this case, serial culture (passage) will provide an adequate amount of MSCs. Furthermore, the MSCs that are obtained from the isolation from fresh tissue need to be collected and stored to be used later on. In this case the MSCs should be cryopreserved.

Cryopreservation is an efficient method for the storage and collection of MSCs, which provides a number of needed cells at the right time for clinical applications. In the process of cryopreservation, MSCs will undergo cryo-injury, but that can be minimized by the use of a variety of cryoprotectants, and slow cooling rate to maintain the functional properties of MSCs including their immunomodulatory properties and multilineage differentiation capabilities [5-7]. One of the most widely used sources of MSCs is bone marrow. Bone marrow MSCs are obtained through bone marrow aspiration procedure. The location of aspiration is

usually the iliac crest and sternum. The MSCs from bone marrow can be cultured to expand them and then can be used for implantation [8, 9].

At present, clinical applications of bone marrow MSCs will lead to allogeneic rather than autologous use. Technically, allogeneic MSCs are able to provide viable cells with good quality that are ready for use in a short time, while autologous MSCs require a longer time as they need to be isolated and expanded , and the quality might not be optimal when they come from an old individual (patient). Moreover, bone marrow aspiration from the patient causes higher morbidity.

The use of bone marrow MSCs in regenerative medicine requires quality control of the product, including viability and replicative senescence data. Replicative senescence usually occurs at high passage, and can be predicted by looking at cell size and population doubling time. A study showed that serial passages caused senescence of umbilical cord MSCs [10].

For the sake of efficacy and safety of bone marrow MSC therapy, it is necessary to do a study to assess the effects of passage on cryopreserved bone marrow MSCs to get an understanding of the effects of serial passages on the aging process of cryopreserved bone marrow MSCs. Therefore, in this study, the effects of serial passages on the aging profile of cryopreserved bone marrow MSCs were evaluated by cell size, population doubling time, cell viability and SA-ß-Gal activity.

Materials and Methods

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

Materials: This study used a bone marrow MSC sample that was cryopreserved in passage one (P1), which was derived from an adult woman.

Procedure: The sample was thawed and recultured to P2. A part of the P2 cells were directly subjected to serial passages to be the once cryopreserved group, while another part was cryopreserved to be the twice cryopreserved group, which was also subjected to serial passages.

Cryopreservation: Cryopreservation medium contained 10% dimethyl sulfoxide (DMSO) in complete medium (40% platelet rich plasma, 1% heparin, 1% penstrep, 1% fungison in alpha-MEM). Cryopreservation was done by gradually decreasing the temperature from room temperature to -190^0 C in 120 minutes by putting the stem cell containing cryo-vial into a planner machine. The stem cells were stored for one month.

Serial Passages: After thawing, the cells were seeded into wells as much as 20.000 cells/cm2, and then 5000 cells/cm2 for the subsequent passages. All cultures were done in ten times repetition (decuplo). Observation of cell growth was done every day and the culture medium was changed every two till three days. Harvest was done when the culture was 70-80% confluent.

Cell Size Assay: After the confluence reached 30%, cells were photographed using an inverted microscope in five visual fields with 40 times magnitude. Fifty fibroblasts were picked randomly for measurement. AxioVision Rel.4.8 was used to measure the cell area.

PDT and Viability Assay: After the cell growth reached 70-80%, cells were harvested by TrypLE Select (Gibco 12563-011), and the viability and PDT were computed starting at P3 for once cryopreserved group, and at P4 for twice cryopreserved group. The formula for calculating PDT was: t/n; n = log (Nh)-log(Ni)/log2**;** t was culture duration (in days), n was population doubling time, Nh was total harvested cells, Ni was total seeded cells.

Senescence Assay: Fixations and stainings for senescent assays were done with Sigma CS0030-IKT. A positive senescent cell will express *senescence associated ß-galactosidase* (SA-ß-Gal), which would be seen as a blue-green color appearing 12-16 hours after incubation. Stained cells were photographed by a camera connected to an inverted

microscope with 100 times magnitude. From each well, five fields were randomly chosen to calculate the number of cells that SA-ß-Gal (+).

Data Collection and Analysis: Statistical analysis was performed in SPSS v.23, and the statistical tests were listed on the table below.

Table 1: Statistical test

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

Statistical comparative analysis was done to compare once and twice cryopreservation groups. Normality test of distributions were done beforehand in order to determine the test that had to be used. Saphiro-wilk test was chosen as the data was less than 50.

Results

Comparison of once and twice cryopreserved bone marrow MSCs in term of PDT, cell size at 30% confluent, viability and senescent cell percentage can be seen in Table 2.

From analysis between the two cryopreservation groups, it was found that significant differences appeared in the viability, PDT, and in 30% confluent cell size, at P5 and P6. In terms of viability, the twice cryopreservation group had a significantly lower viability compared to the once cryopreservation group at the sixth passage ($p = 0.022$), but had a higher viability in the fourth and fifth passage. Overall PDT in the twice cryopreservation group took a longer time than the once cryopreservation group. In term of cell size, the twice cryopreservation group had a significantly larger cell size than the once cryopreservation group. In both cryopreservation groups bone marrow MSCs began to show more than 5% senescence at P6, where senescence percentage was not significantly different between the two groups.

Table 2: Differences between once and twice cryopreserved bone marrow MSCs

Variables	First Cryopreservation		Second Cryopresrvation		
	Mean (±SD)	Normality	Mean (±SD)	Normality	P Value
Viability (%)					
P3	92.64 (3.46)	0.56			
P4	91.04 (4.55)	0.87	93.51 (2.21)	0.471	0.223*
P5	93.54 (1.86)	0.7	96.64 (2.75)	0.122	0.01*
P6	92.42 (5.66)	0.187	87.44 (1.12)	0.371	0.022*
Population doubling time					
P3	1.845 (0.163)	0.053			
P4	2.336 (0.521)	0.084	2.499 (0.364)	0.445	0.429*
P5	1.626 (0.123)	0.412	2.089 (0.145)	0.589	<0.001*
P6	10.359 (7.37)	0.001	3.643 (0.114)	0.142	<0.001**
Cell size 30% (µm2)					
P3					
P4	1985.87 (95.29)	0.069			
P5	1794.35 (212.65)	0.855	1965.22 (272.52)	0.423	0.136*
P6	1600.65 (122.01)	0.787	1872.26 (95.73)	0.531	<0.001*
	1621.07 (108.87)	0.301	2005.43 (105.68)	0.025	<0.001**
% Cell *Senescence* (%)					
P3					
P4	0.573 (0.15)	0.347			
P5	1.18 (0.29)	0.631	1.365 (0.171)	0.956	0.115*
P6	0.229 (0.349)	0.314	3.10 (0.318)	0.464	<0.001*
	10.72 (1.57)	0.68	9.564 (0.62)	0.109	0.052*

*Student T test was done in normal data

** Mann-whitney Test was done in abnormal frequency data

Significance test between passages in once and twice cryopreservation group can be seen in Table 3 and 4 respectively.

In the 30% confluent cell size in the once cryopreservation group, we found a significant reduction in P6 compared to P3 ($p < 0.001$) (Table 2 and 3), while at the twice cryopreservation group, cell size increase was not significant in comparison between the P4 and P6 (Table 2 and 3).

The percentage of senescence cells in the once cryopreservation group was significantly increased at P6 compared to P3, P4 and P5 (Table 2 and 3). Similarly, in the twice cryopreservation group, there were significant increases in percentage of senescence cells in P6 when compared P4 and P5 (Table 2 and 4).

Table 3. Significance test between passages of once cryopreservation of bone marrow MSCs using *multiple comparison* ANOVA and Tukey HSD

First Cryopreservation

PDT

	P4	P5	P6
P3	0.991		0.000
P4		0.973	
P5			0.000

30% confluent Cell Size

	P4	P5	P6
P3	0.024		0.000
P4		0.022	
P5			0.988

Viability

	P4	P5	P6
P3	0.824		0.999
P4		0.537	
P5			0.929

Persentage of senescence cells (+)

	P4	P5	P6
P3	0.357		0.000
P4		0.023	
P5			0.000

Table 4: Significance test between passages of twice cryopreservation bone marrow MSCs using *multiple comparison* ANOVA and Tukey HSD

Second Cryopreservation

PDT

	P5	P6
P4	0.014	0.000
P5		0.000

30% confluent Cell Size

	P5	P6
P4	0.480	0.869
P5		0.232

Viability

	P5	P6
P4	0.003	0.000
P5		0.000

Persentage of Senescence cells (+)

	P5	P6
P4	0.000	0.000
P5		0.000

Discussion

Cell Size

In this study, we found that the size of the bone marrow MSCs was bigger in twice compared to once cryopreservation group. Our result was in line with the findings of Legzdina et al., and Cui et al. [11, 12]. Legzdina et al. [11] explained that the senescent cells, would undergo morphological changes to become larger and showed increased heterogeneity in form, which might be irregular fibroblastic, irregular, or star shape, flat, and showed granular cytoplasm. However, Legzdina's study was performed on initial passages (P3 and P4) with a duration of cryopreservation of 1.5 to 3.5 years and the age of the donors was 27-61 years old [11]. In this study, an increasing in cell size along cryopreservation can be explained by the study of Cui et al. [12] that showed that by performing multiple cryopreservations, there were more death cells, so that the cells

that were still viable had to do more division, which in turn might produced more aging cell due to DNA damage and shortening of the telomeres. In our study, the size of the cells got bigger as the number of cryopreservation increases. We used DMSO as cryoprotectant, while Cui's research used a cryopreservation method with the addition of permeable (0.5 M DMSO) and non-permeable (0.2 M trehalose) cryoprotectants.

Viability

We found that the viability of bone marrow MSCs decreased significantly in the twice cryopreservation group only at P6. Overall, MSCs In the once cryopreservation group, viability varied between passages, but no significant difference was observed between passages (Table 3). In the twice cryopreservation group, the viability differed significantly between passages (Table 4). However, viability was more than 85 % in all passages of both groups, thus the difference might be regarded as normal variation.

The decreasing cell viability may be caused by the amount of cryopreservation that was done. Lambertyn et al. [13] stated in her study that cryopreservation decrease cell viability, but by adding a cryoprotectant it can be minimized. Cui et al. [12] revealed that even with the addition of cryoprotectants, cryo-injury still occurred, causing damage and cell death. In this study, the second cryopreservation that was performed provided a bigger cryo-injury effect than in the first cryopreservation. The method used to measure viability was tryphan blue dye exclusion. One limitation of this study is that we did not use any other method, for example 3- (4,5-dimethylthiazol-2-yl) -2-2-diphenoltetrazolium bromide (MTT).

Trypan blue concentration and incubation time during the counting process might affect the viability of the examination in this study. Kwok et al. [14] reported in his study that high concentrations of trypan blue (4 mg / mL) may cause toxicity and was indicated by a decrease in viability and disruption of gene expression associated with apoptosis and cessation of growth. Trypan blue concentration used in this study was 0.4%, so did not considered to be toxic.

Population Doubling Time

In this study, we found that P4 and P5 population doubling times of bone marrow MSCs increased in the twice compared to once cryopreservation group. Moreover, there was an increased of PDT mainly at P6 of the once cryopreservation group (Table 2). Similarly, in the twice cryopreservation group, the PDT was also increased in P6 compared to P4 and P5 (Table 2 and 4).

Schellenberg et al. [15] revealed various factors may affect PDT, among others were duration of cell culture, number of cells that were seeded, harvested cell numbers, cell growth phase at harvest (log phase), as well as the heterogeneity of the cells that some were already experiencing replicative aging.

Fossett and Khan [16] in the study mentioned that a low density seeding (100 cells / cm^2) had a proliferation time of 4.1 days, earlier than high density seeding (5000 cells / cm^2). Proliferation was low in the cells with high density planting, which may be due to inhibition contact, whereas low density seeding will provide a high intake of nutrients to every cell. In this study, there was no PDT assay of senescent cell, which was a limitation of this study.

Senescence

Evaluation of senescence of bone marrow MSCs was done by calculating the percentage of senescence cells. In bone marrow MSCs, cell senescence occurred in the sixth passage (>5%), both in the once and twice cryopresercation groups.

In his study, Mamidi et al. [17] reported that the percentage of senescence cell of bone marrow MSCs increased at the sixth passage. Choi et al. [18] said that proliferation of MSCs would decline in long-term passage. Decreasing the proliferation caused more cell division to achieve confluence, therefore DNA damage occured more rapidly which eventually led to the Hayflick limit and the aging of cells occurs. Goh et al. [19] found that cryopreservation of mesenchymal stem cells decreased the adhesion efficiency that led to proliferation disturbance and senescent of the stem cells.

Conclusion

There is a passage effect on the senescence of cryopreserved bone marrow at passage six and the multiple passage influences the senescence, viability, proliferation (PDT) and cell size. This study needs to be followed by adding a research with more samples with different characteristics (age, race, and BMI). Numbers of cryopreservation may be a determining factor.

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