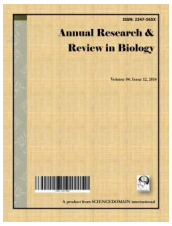

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Comparison of Various Solutions for Temporary Storage of Umbilical Cord Derived Mesenchymal Stem Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Author JAP designed the study, wrote the protocol, managed the literature searches, revised the draft, prepared the figures and tables. Authors SIK, RA and OEY compiled the data, calculating viability, PDT, performed the statistical analysis and wrote the first draft. Authors MR and RA performed the culture and collecting the data. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine whether physiologic saline, phosphate buffered saline (PBS) and high glucose Dulbecco's modified Eagle's Medium (DMEM-HG) were suitable as temporary storage solution.
Study Design: In vitro experimental analytical study.

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 # Equally contributed

Place and Duration of Study: Stem Cell Medical Technology Integrated Service Unit, RSCM/Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia, from August through December 2016.
Methodology: We did viability assessments at various time points, namely after 0, 3, 6, 24, 48, 72, 96, and 168 hours in the three storage solutions. For population doubling time (PDT) assessments the cells were re-cultured after various time points in the tested solutions. All viability and PDT assessments were done in four replications. Furthermore, for all storage solutions, differences between the various time points in terms of viability and PDT were compared and tested by ANOVA or Kruskal-Wallis test.
Results: Viability was >70% up to 72 hours in PBS and physiologic saline, and up to 96 hours in DMEM-HG. Decrease in viability began to be significant compared to initial viability after 6, 24, and 72 hours in PBS, physiologic saline, and DMEM-HG respectively. Increase in PDT began to be significant compared to initial PDT after 3, 6, and 24 hours in PBS, physiologic saline, and DMEM-HG respectively.
Conclusion: For our UC-MSCs, DMEM-HG is the best temporary storage solution, and storage should not exceed 24 hours.

Keywords: Umbilical cord; mesenchymal stem cell; storage solution; viability; population doubling time.

ABBREVIATIONS

MSCs : Mesenchymal stem cells
UC-MSCs : Umbilical cord derived mesenchymal stem cells
PBS : phosphate buffered saline
DMEM-HG: High glucose Dulbecco's modified Eagle's Medium
PDT : Population doubling time
CFU : Colony forming unit

1. INTRODUCTION

Stem cells have gained much popularity nowadays in the field of regenerative medicine for its remarkable potential in treating various diseases [1], and are potential to be developed as biopharmaceuticals. There are many types of stem cells, such as neural stem cells, hematopoietic stem cells, mesenchymal stem cells (MSCs), etc. Each type of stem cell originates from different body regions and has variable differentiation capacity. For instance, MSCs can differentiate into cells of chondrogenic, osteogenic and adipogenic lineage [2]. Recently, MSCs are preferable as they have low immunogenicity as they are human leukocyte antigen (HLA) class II negative [2], which means that they are better tolerated by the host in a transplant. MSCs can differentiate to different cell types in vitro, but most of their effects were due to paracine secretions, and modulation of the immune system [1,2].

Umbilical cord tissue as a source for multipotent mesenchymal stem cells has many advantages over other sources such as bone marrow and adipose tissue. Since umbilical cord tissue is a waste of the parturition process, their collection is not invasive. Additionally, the mesenchymal stem cells derived from umbilical cord tissue are thought to have lower immunogenicity, and immune-modulator properties, which allow them for allogeneic usage [3].

We have developed a simple isolation method for umbilical cord derived mesenchymal stem cells (UC-MSCs) by multiple harvest explants method [4]. The UC-MSCs were used in a case of infected non-union femoral shaft fracture with a 12 cm bone defect [5]. In regenerative medicine, various solutions have been used as vehicle for stem cells, such as physiologic saline [6-8], phosphate buffered saline [9-10], Earle's balance salt solution [11], etc. The various vehicles were used for bone marrow (BM) mononuclear cells [6], human BM MSCs [7,8,10], rat BM MSC [9, 11], human dental pulp MSCs, and adipose tissue MSCs [10], but cell endurance in these solutions was not evaluated [6-11]. When stem cells are transported from the lab to the place of treatment, these vehicles serve as temporary storage solution. It is not known, which solution is the best temporary storage solution for our UC-MSCs. Therefore, the aim of this study was to determine whether physiologic saline, phosphate buffered saline and high glucose Dulbecco's modified Eagle's Medium (DMEM-HG) were suitable as temporary storage solution of our UC-MSCs. Suitability as temporary storage solution was assessed using viability and population doubling time (PDT) after various time intervals

in the three storage solutions. The most suitable solution can be used as temporary storage of our UC-MSCs in clinical setting.

2. METHODOLOGY

This in vitro experimental analytical study was conducted in Stem Cell Medical Technology Integrated Service Unit, RSCM/Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia, from August through December 2016. Ethical clearance (no. 157/H2.F1/ETIK/ 2014) was obtained from Faculty of Medicine Universitas Indonesia Ethical Commitee in 2014 for a research project on umbilical cord derived mesenchymal stem cells, and was amended to get prolonged.

2.1 Sample

The sample was cryopreserved pre-characterized human UC-MSCs that were stored in Stem Cell Medical Technology Integrated Service Unit, RSCM/Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia.

2.2 Procedures

Cryopreserved UC-MSCs were thawed and recultured in three 25 cm2 tissue culture flasks in complete medium as previously described [12]. At 80% confluence, the cells were harvested pooled, and counted. Cell suspension was divided in three equal numbers and tranfered into three Eppendorf tubes. Then the tubes were centrifuged, and the cell pellets were each resuspended in 1 mL of tested solution, namely, physiologic saline, PBS, or DMEM-HG. Viability and PDT of the cells in the three tubes was assessed and regarded as initial viability and PDT. For PDT assessment, 10,000 viable cells were seeded in four wells of a 24 well plate, cultured until confluent, and harvested. Time to confluence and cell number at harvest were noted, to calculate the PDT. Then, the cells in all three tubes were divided into eight equal amount and tranfered into new Eppendorf tubes, to yield 24 tubes, which consisted of 3 sets of 8 tubes. The 8 tubes in each set were used at various time points (0, 3, 6, 24, 48, 72, 96, and 168 hours). The 3 sets of tubes contained cells in physiologic saline, PBS, or DMEM-HG. The amount of solution in the 24 tubes was all made 1 mL with respective solutions, and stored in 4^{o}C until used.

Further, for each tested solution, viability assessments were done at various time points, namely after 0, 3, 6, 24, 48, 72, 96, and 168 hours in tested solutions. For PDT assessments the cells were re-cultured after various time points in tested solutions. All viability and PDT assessments were done in four replications.

2.2.1 Viability assessment

For viability assessment, viable and dead cells were distinguished by Trypan blue dye exclusion method and viable and dead cell numbers were counted by a Neubauer hemocytometer. For all type of solutions and various time points, viable and dead cell counts were done in four replications. Viability of the cells was computed by dividing viable cell number with total (viable and dead) cell number.

2.2.2 Population doubling time calculation

Population doubling time was computed using the equation below [13]:

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

Where:

- ΔT = length of culture time
- NH = number of total harvested cells
- NI = initial seeding number

2.3 Data collection and Analysis

Data collected were viable and dead cell number before culture and at harvest after 0, 3, 6, 24, 48, 72, 96, and 168 hours in various storage solutions. Viability and PDT were computed, and means and standard deviations of viability and PDT at various time points were calculated, and presented as viability and PDT curves.

Further, for all storage solutions, differences between the various time points in terms of viability and PDT were compared by either ANOVA when the data was suitable or Kruskal-Wallis test when the data was unsuitable for parametric analysis (non normal distribution and nonhomogenous variance). When there were significant difference, determination of difference between time points were done by posthoc analysis, i.e by Tukey following ANOVA, or Dunn following Kruskal-Wallis test. Data analysis was done using statistical analysis software, SPSS 20.0.

3. RESULTS AND DISCUSSION

Viability of UC-MSCs after 3, 6, 24, 48, 72, 96, and 168 hours suspended in DMEM-HG, physiologic saline and PBS can be seen in Fig. 1. Table 1 shows the p values of differences in viability after 3, 6, 24, 48, 72, 96, and 168 hours suspended in DMEM-HG, physiologic saline and PBS compared to initial viability.

3.1 Viability

Our result showed that decrease in viability began to be significant compared to initial viability after 6, 24, and 72 hours in PBS, physiologic saline, and DMEM-HG respectively (Figure 1, Table 1). However, after 72 hours in the three tested solutions, viability was still >70%, which shows that the cells still met the criteria of FDA to be used in cell therapy [14]. Regarding cell viability in physiologic saline, our results are in line with the results of other studies [15-17]. Ra et al. [15], who assessed the viability of MSCs after suspension in physiologic saline solution under the same temperature as in our study showed an average viablity of 85.4% after 72 hours. Veronesi et al. [16] compared complete medium (8% platelet lysate containing αMEM), 4% human serum albumin containing physiologic saline (pH 7.0), and physiologic saline alone. They reported that after 18 hours in tested storage solutions at 4℃, complete medium showed the best viability (88%), while the other two showed comparable results with a viability of about 83%. In this study, dead cells were assessed by propidium iodide (PI) and flow cytometry, which was comparable to Trypan blue exclusion method [16]. Sohn et al [17] compared physiologic saline and 5% dextrose solution and found that saline was better than 5% dextrose, and that after 6 hours in saline at 4^{o}C, viability that was assessed by Anexin V/PI was >85% .

Various studies used various solutions as vehicle for cell transplantation in various conditions, but those studies did not check the viability and attachment/proliferation capacity of the cells after a certain period in the respective solutions [6-11]. Therefore, we could not compare our results with the results of those studies. Our results in PBS were in line with the results of Muraki et al. [18] who stored MSCs in PBS at 4℃ for up to 24 hours and found that the viability was 81%, which was better than storage at 24℃ and 37℃, where the viability was 70% and 62% respectively.

3.2 Population Doubling Time (PDT)

Our result showed that increase in PDT began to be significant compared to initial PDT after 3, 6, and 24 hours in PBS, physiologic saline, and DMEM-HG respectively (Figure 2, Table 2). This result shows that although there was no significant decrease in cell viability after 3, 6, and 24 hours in PBS, physiologic saline, and DMEM-HG, the viable cells already lost their attachment and proliferation capacity, which caused significant increases in PDT. Our result was in line with the result of Sohn et al, where viability did not correspond with attachment and proliferation capacity, which was assessed by colony forming unit assay [17]. Sohn et al [17] showed that colony forming unit (CFU), which correspond to the number of cells with attachment and proliferation capacity, was decreased to 50% after 2 hours of storage in physiologic saline at 4^{o}C, though the viability was still > 90%. Therefore, PDT might be used as surrogate marker of attachment and proliferation capacity.

The discrepancy between the timing of viability decrease and PDT increase might be due the use of Trypan blue dye exclusion method to assess viability in this study, which was the limitation of this study. Another limitation was the study was done on cells, which derived from one sample.Trypan blue dye exclusion method is an established method to distinguish viable from dead cells, and was used in many studies [2,12, 13,19-21]. However, Trypan blue can not distinguish dead cells from cells in the process of dying, and give a higher viability value compared

Table 1. *P* values of viability differences between various time points and initial viability

Storage solution	*P* values at various time points in solution						
	3h	6h	24h	48h	72h	96h	168h
DMEM-HG	0.386*	0.248*	0.248*	0.083*	0.043*	0.021*	0.021*
Saline	0.774**	0.061**	0.008**	0.010**	0.000**	0.000**	0.000**
PBS	0.083*	0.043*	0.021*	0.021*	0.021*	0.021*	0.021*

*h= hours, *Dunn posthoc after Kruskal-Wallis test, **Tukey posthoc after ANOVA test*

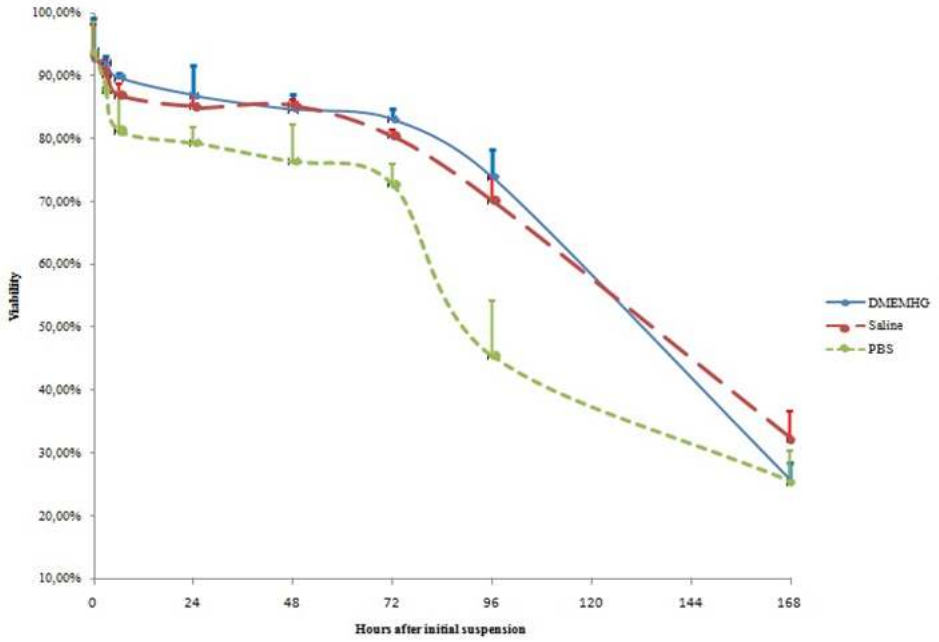

Fig. 1. Viability of UC-MSCs at various time points in various storage solution

Table 2. *P* values of PDT difference between various time points and initial PDT

Storage solution	*P* values at various time points in solution (hours)*						
	3h	6h	24h	48h	72h	96h	168h
DMEM-HG	0.564	0.248	0.021	0.020	0.021	0.021	0.000
Saline	0.243	0.043	0.021	0.021	0.021	0.021	0.021
PBS	0.043	0.021	0.021	0.021	0.021	0.021	0.021

*h= hours, *Dunn posthoc after Kruskal-Wallis test*

to combination of Annexin V and PI staining [22]. Dying cells lose some of their functions [22], including attachment and proliferation capacity, thus the remaining viable and functional cells should proliferate more to yield 80% confluence, which cause a longer time to harvesting, and a higher PDT. Therefore, in this study, increase in PDT appeared faster than decrease in viability. However, viable cells that were assessed by Anexin V/PI also showed decrease attachment and proliferation capacity [17]. This shows us that viable cells may be non-functional.

3.3 Clinical Relevance

Based on FDA guidelines [14], UC-MSCs are still eligible for cell therapy up to 72 hours in physiologic saline or PBS, and up to 96 hours in DMEM-HG (Fig. 1). However, PDT began to be significant different from initial PDT after 3, 6 and 24 hours in PBS, physiologic saline, and DMEM-HG respectively. This fact is due to the contents of DMEM-HG that are far richer as compared to PBS or physiologic saline. DMEM-HG not only has an isotonic ionic concentration that is similar to human interstitial fluid, it is also rich in amino acids, vitamins and glucose [23], which are supportive to cell metabolism for a certain period, while physiologic saline and PBS only offers an isotonic condition. However, after 168 hours in DMEM-HG, UC-MSCs ceased to proliferate (Fig. 2), which might be due to exhaustion in cell metabolism. There was no other available data concerning storage in DMEM-HG, but Garvican et al. [24] used equine bone marrow MSCs that were stored in 10% fetal bovine serum containing DMEM at 4-8℃ and found that viability was 67%. As PDT might be used as surrogate marker of attachment and proliferative function, for clinical use, DMEM-HG that could maintain the PDT for the longest time is better than physiologic saline and PBS. Moreover, as

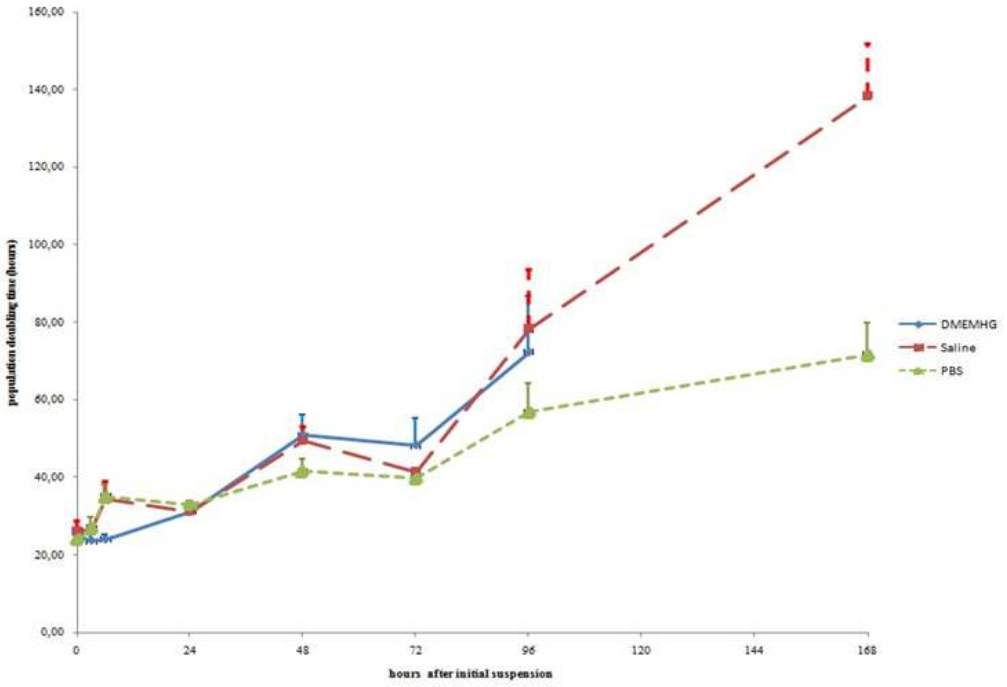

Fig. 2. Population doubling time of UC-MSCs at various time points in various storage solution

PDT was significantly increased after 24 hours that was in line with reduced functionality, the use of the cells after 24 hours needs an increase in cell number.

4. CONCLUSION

Taken both viability and PDT into account, DMEM-HG is the best temporary storage solution, and storage should not exceed 24 hours, otherwise the cell number should be increased.

CONSENT

This study used cryopreserved umbilical cord derived mesenchymal stem cells from a previous study, which obtained the umbilical cord from a donor that has given their informed consent.

ETHICAL APPROVAL

Ethical clearance (no. 157/H2.F1/ETIK/ 2014) was obtained from Faculty of Medicine Universitas Indonesia Ethical Commitee in 2014 for a research project on umbilical cord derived mesenchymal stem cells, and was amended to get prolonged.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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