

The Potential of Basal Medium Astemporary Prolong Culture of Umbilical Cord Derived Mesenchymalstem Cells

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To compare the viability and characteristics of umbilical cord mesenchymal stromal cells (UC-MSCs) when they were kept in culture in three types of α MEM after confluence for various time points. Viability and characteristic assessments were done after keeping UC-MSC confluent cultures for 0, 24, 48, 72, and 96 hours in α MEM with glutamax (Himedia), and without glutamax (Himedia or Gibco). There were thirty cultures, which each ten of them were kept in the three types of basal media, and for each time point and type of basal medium, two flasks were harvested. All viability and characteristic assessments were done after harvest. Viability assessments were done in duplicate, while characteristics assessment by flow cytometry for CD73, CD90 and CD105 were done only once. Further, differences between the various time points in terms of viability and CD73, CD90 and CD105 percentage after various time points were compared and tested by appropriate statistical analysis. Viability was >70% upto 72 hours and 48 hours when the cells were kept in Himedia glutamax containing α MEM and α MEM without glutamax respectively. Flow cytometry showed that CD73, CD90 and CD negative percentage did not differ to initial percentage, but after 24 hours CD105 was slightly decreased to > 60% in Himedia glutamax containing α MEM, while in the two α MEM media without glutamax the percentages were below 40%. For our UC-MSC culture, glutamax containing α MEM is better compared to α MEM without glutamax as temporary storage solution, and storage should be restricted to 24 hours.

Keywords: umbilical cord, mesenchymal stem cell, storage solution, viability, population doubling time.

We have isolated and propagated umbilical cord derived mesenchymalstromal/stem cells (UC-MSCs) using the multiple harvest explant

method, 1 and our UC-MSCs met the standards that are set by International Society for Cell Therapy (ISCT) in term of their surface markers and

their capacity to differentiate into three lineages, namely adipogenic, chondrogenic, and osteogenic lineages.² Umbilical cord was chosen because it was a waste of the parturition process and their collection was not invasive; therefore, it has many advantages over bone marrow (BM) or adipose tissue (AT), which are previously developed and frequently used as MSC sources. Moreover, UC-MSCs have immune-modulator properties, and are believed to have lower immunogenicity compared to BM or AT MSCs, which privilege them for allogeneic use.³

Our UC-MSCs have been used in clinical trials that are on-going, and after harvest we stored the UC-MSCs in a temporary storage solution at 4-8°C before application. A study showed that after harvest, our UC-MSCs could be suspended in high glucose Dulbecco's modified Eagle's Medium (DMEM-HG) until 96 hours, which was superior to physiologic saline, and phosphate buffered saline (PBS). However, beyond 96 hours, DMEM-HG stored UC-MSC suspension completely lost their attachment and proliferation capacity, while physiologic saline and PBS stored UC-MSCs still retained some of their attachment and proliferation capacity. Moreover, beyond 96 hours, decline in viability of DMEM-HG was faster compared to physiologic saline stored UC-MSCs.⁴ DMEM-HG contains high glucose, as well as amino acids, vitamins and isotonic ionic concentration, which is similar to human interstitial fluid.⁵ High glucose containing solution might be supportive to cell metabolism for a certain period, but in longer period might trigger exhaustion in cell metabolism, which led to proliferation arrest.⁴ Therefore, normal glucose basal medium might be more suitable in maintaining our UC-MSC viability.

Keeping harvested cell suspension in storage solution is often needed due to incompatible harvesting time and stem cell application schedule, either in clinical or preclinical setting, such as in organoid culture, where various types of cells are needed. Another approach to keeping harvested cell suspension in storage solution is keeping near confluent cultures in serum free basal medium until application schedule (prolong culture). Therefore, the aim of this study was to compare the viability and characteristics of our UC-MSCs when they were kept in culture in three types of α MEM after confluence for various time points.

MATERIALS AND METHODS

This *in vitro* analytical study was conducted in Stem Cell Medical Technology Integrated Service Unit, RSCM/Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia, and Stem Cell and Tissue Engineering Research Center, Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine Universitas Indonesia. Ethical clearance for this study was obtained from Ethical Committee of the Faculty of Medicine Universitas Indonesia in 2018 (no. 0144/UN2.F1/ETIK/2018).

Sample

The sample was pre-characterized human UC-MSCs at passage-4, which was isolated by the multiple harvest explants method, cryopreserved, and stored in liquid nitrogen.¹ The sample was obtained from Stem Cell Medical Technology Integrated Service Unit, RSCM/Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia.

Procedures

Cryopreserved UC-MSCs were thawed quickly and recultured in a 25cm² tissue culture (T25) flasks in 10% platelet lysate containing complete medium as previously described.⁶ Further, at 80-90% confluence, the cells were harvested and counted.

Comparison of three types of basal media

Cell suspension was divided in three equal volumes and the cell pellets were each resuspended in three types of 10% platelet lysate containing complete media. The three complete media differed in the basal medium, namely glutamax containing α MEM (Himedia AL081G), and α MEM without glutamax (Himedia AL080A or Gibco 41061-029). Each cell suspensions in the three kinds of complete media were recultured into ten T25 flasks to yield thirty T25 flasks. The flasks were observed every day. When the cultures reached 80-90% confluence, complete medium was changed with the respective basal medium, except for two flasks from each basal medium, which was directly harvested and the results were labeled as the results at 0 hour (initial viability). The other flasks, two flasks from each basal medium were kept for 24, 48, 72, and 96 hours in 37°C, and harvested.

After harvesting, viability and characteristic assessments were done. Viability assessments were done in two replications, while characteristic assessments by measuring the CD73,

CD90, and CD105 by flowcytometry were done only once.

Data collection and analysis

Collected data were viability and percentage of CD73, CD90, and CD105 at harvesting after 0, 24, 48, 72, and 96hours in the three types of basal medium. Viability in the three types of media after various time points were tabulated; mean and standard deviation were calculated and presented as viability curves. Percentage of CD73, CD90, and CD105 in the three types of media after various time points were tabulated, and presented as line graphs. Further,

differences between the various time points and the three types of basal media in terms of viability were compared by either ANOVA or Kruskal-Wallis test when the data was unsuitable for ANOVA. Correlation between viability and time were done. All analysis was done using statistical analysis software (SPSS 20.0).

RESULTS AND DISCUSSION

By the time, the cell viability was decrease gradually. The gradual decrease of cells' viability was slowest in the glutamax containing

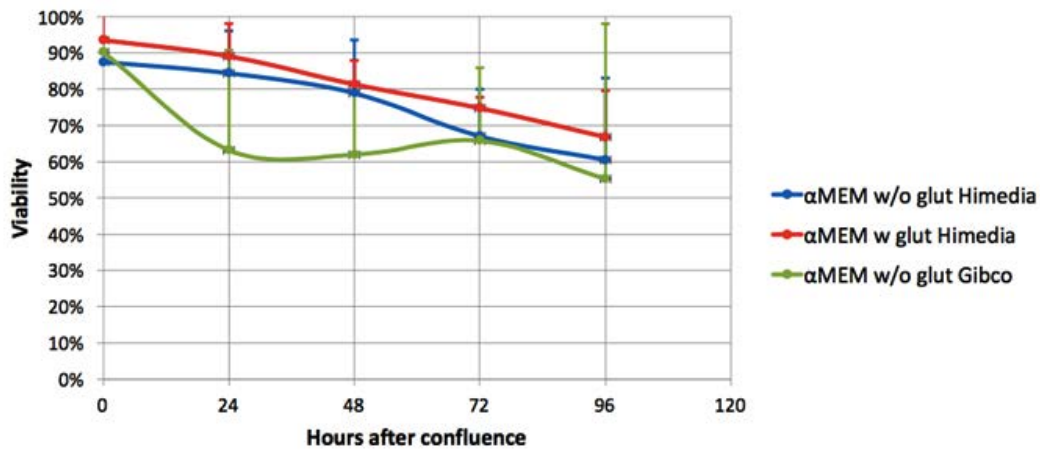


Fig. 1. Viability of UC-MSCs at various time points in glutamax containing basal media (Himedia), and basal media without glutamax (Himedia or Gibco)

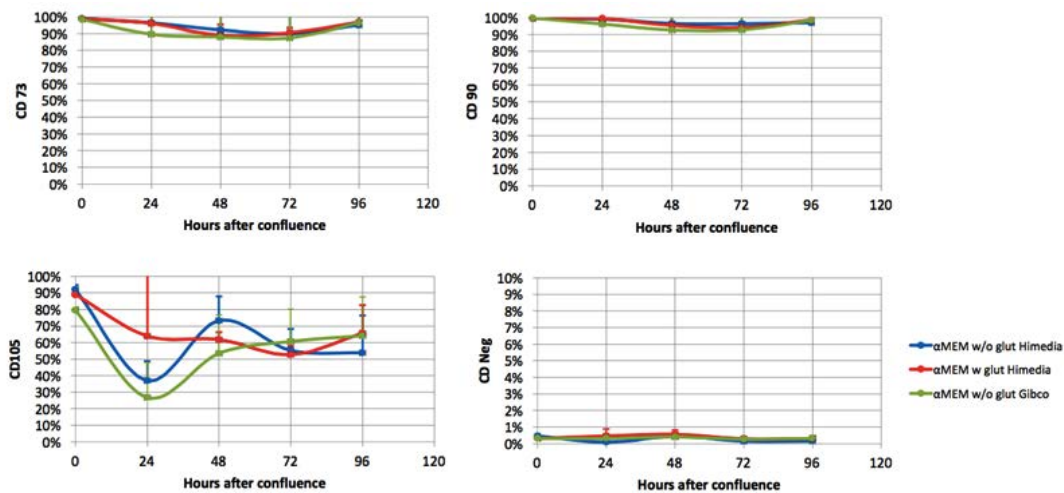


Fig. 2. Percentage of CD73, CD90 and CD 105 at various time points in glutamax containing basal media (Himedia), and basal media without glutamax (Himedia or Gibco)

MEM (Himedia), followed by MEM without glutamax(Himedia), and MEM without glutamax (Gibco) basal media. In the first 24 hours after confluence, the cells' viability in MEM without glutamax(Gibco) basal media has decreased until less than 70%; whereas, in the MEM without glutamax(Himedia) and in the glutamax containing MEM (Himedia) it decreased in the 72 hours and 96 hours after confluence respectively (Figure 1). Our data were normally distributed, but the variances were nonhomogenous, thus unsuitable for ANOVA. Kruskal Wallis test showed that there were no difference in viability between the three tested basal media, and between different time points ($p=0.053$).

Characterization of cells using cluster of differentiation (CD) protein markers showed an interesting picture. The expressions were kept stable in CD73, CD90 and CD105, however, it was dynamic in the CD105 expression (Figure 2). During the time, expression of CD 90 was constantly >90%, and the CD105 expression was constantly below 1%. The expression of CD73 in the three medium was comparable, although in basal media without glutamax (Gibco) it had dropped below 90%. The expression of CD105 could not be maintained in 90% or above. It dropped significantly in the first 24 hours after confluence in the three basal media (Figure 2).

Figure 1. Viability of UC-MSCs at various time points in glutamax containing basal media (Himedia), and basal media without glutamax (Himedia and Gibco) - here

Figure 2. Percentage of CD73, CD90 and CD 105 at various time points in glutamax containing basal media (Himedia), and basal media without glutamax (Himedia and Gibco) -here

Our result showed that there were no significant decreases in viability between the three tested basal media. However, when we refer to FDA criteria where viable cells for cell therapy should be at least 70%, not all of the various time points in those media met the criteria. Before 72 hours after confluence, only in both Himedia with and without glutamax containing MEM the viabilities were >70%. In MEM without glutamax (Gibco), viability even decreased below 70% after 24 hours (Figure 1)

Characterization after various time points

in the three kinds of basal media showed that CD73, CD90, and negative mesenchymal stem cell markers did not differ between the various time points, and media. However, CD 105 dropped below 80% after 24 hours in all media (Figure 2). CD105 (endoglin), which in this study was > 60% after 24 hours of incubation in glutamax containing MEM, is one of the surface markers on MSCs that should exceed 90% according to ISCT guideline. However, this guideline was set for bone marrow derived MSCs. Another guideline that was set by International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) stated that for adipose tissue derived stem cells, CD105 should be >80%.

CD105 functions as an accessory co-receptor for transforming growth factor beta (TGF β), which can be found on syncytiotrophoblasts, fibroblast, haematopoietic progenitor cells, and endothelium of microvascular in normal tissues. Its expression is elevated in endothelial cell proliferation, as well as endothelium and stromal cells of solid malignancies, and thus has been suggested to play a role in angiogenesis and neovascularization. Moreover, as TGF β co-receptor CD105 might play a role in cell adhesion, proliferation, migration, and differentiation.

In this study, reduced CD105 after 24 hours might interfere with MSC's differentiation capacity and stemness. In this preliminary study we did not examine MSC's differentiation capacity or population doubling time and fibroblast colony forming unit as surrogate of stemness, which is a limitation of this study. Therefore, though cell viability still met FDA criteria for cell therapy, after 72 and 48 hours in both Himedia glutamax containing MEM and MEM without glutamax respectively, their functions might be reduced after 24 hours.

Keeping cells in basal media without serum for a certain period is one of the various methods to produce conditioned media rich in growth factors, which was used to treat various pathological conditions. Some studies used hypoxic conditions to produce the conditioned media to increase growth factor secretion. Moreover, normal stem cell niche in tissues is believed to be hypoxic. Therefore, for future direction, studies on keeping cell culture in basal medium in hypoxic

condition that might prolong the time of storage without interfering cell quality need to be done.

Prolonged culture in basal medium for limited time is very useful when discrepancy of time between cell availability and application schedule is short, either for application in clinical setting or organoid research that needs various types of cells that should be ready in a certain time. Discrepancy between cell availability and application schedule can be managed by cryopreservation, but cryopreservation needs re-culture, which at least needs 7 days to provide cells for application. Moreover, the media after short time storage in hypoxic condition can be utilized and tested as therapeutic agents for various pathological conditions.

An alternative instead of to keep cells in culture is to harvest the cells and then keep the cells in a storage solution. We developed a simple processing method to produce adipose tissue derived mesenchymal stem cells (AT-MSCs), 11 and the best storage solution for our AT-MSCs after harvesting was physiologic saline. 12

In conclusion, taken both viability and flowcytometry characteristics into account, for near confluent UC-MSC cultures, glutamax containing α MEM is the best prolong culture solution, and prolong culture should be restricted to 24 hours.

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