



## Simple production method of umbilical cord derived mesenchymal stem cell using xeno-free materials for translational research

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### ABSTRACT

Umbilical cord (UC) is a very promising source of mesenchymal stem cells (MSCs) for allogeneic use, as it can be collected easily without side effects to the donor. In this study, we developed a simple production protocol to get UC-MSCs using xeno-free material to provide safe MSCs for translational research in regenerative medicine. In this study, we used multiple harvest explant method to isolate the MSCs. The medium for isolation and propagation was 10% platelet concentrate (PC) containing alpha MEM. Confluent cultures were harvested, combined and counted. Primary cultures were expanded in T25 flasks (seeding around 5000/cm<sup>2</sup>) to passage-1 (P-1) and P-2, and the results of P-1 and P-2 cultures were counted. From 5 cm of umbilical cord, we did explant culture in four 24 well plates. The number of harvest per well ranges from 0-5 times i.e. no harvest= 6, once= 20, twice= 34, three times= 27, four times=8, and five times= 1 well(s), respectively. Therefore, we harvested a total of 206 times from the 96 wells, and got a total of 3,595,600 cells from the primary culture. If we use all of the cells from the primary culture for expansion, we will get a total of 47,809,462 cells in passage-1, a total of 1,022,607,122 cells in passage-2. In conclusion, UC-MSCs can be isolated and expanded easily in 10% PC containing alpha MEM, and is suitable to fill the demand of allogeneic MSCs for patient use.

**Keywords:** umbilical cord, MSC, PC, explant method, multiple harvest

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### INTRODUCTION

Production of safe stem cells, in particular mesenchymal stem cells as bio-pharmaceutics, is very important in regenerative medicine. Mesenchymal stem cells have immunosuppressant properties *in vitro* and *in vivo*, and low immunogenicity [1]. Moreover, a meta-analysis of MSC using clinical trials, where some of the clinical trials used unmatched allogeneic MSCs, showed no serious adverse effect [2]. Therefore, MSCs might be regarded safe for allogeneic use. However, production procedure that uses fetal bovine serum (FBS) may be harmful to patients as xenomaterial in FBS can be incorporated into the cells and difficult to be eliminated [3].

Umbilical cord (UC) is a very promising source of mesenchymal stem cells (MSCs) for allogeneic use, as it can be collected easily without side effects to the donor. Moreover, UC-MSCs can be expanded more compared to bone marrow or other adult tissue derived MSCs [4], and thus they are more readily available for allogeneic use.

Previous studies isolated MSCs from UC tissue using enzymatic processings [5, 6] or explant method that was harvested once [7, 8, 9]. We have developed a multiple harvest method, which can harvest the explant more than once. However, in the original multiple harvest study, we put three pieces of UC tissue in a well, and observed that the explants did not became confluent at the same time [10]. Therefore, in this study, we modified the method and developed a simple production protocol to get UC-MSCs using xeno-free material to provide safe MSCs for translational research in regenerative medicine.

## EXPERIMENTAL SECTION

This descriptive *in vitro* study was conducted in Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital - Faculty of Medicine Universitas Indonesia from April through July 2014. Ethical clearance for this study was obtained from Ethical Committee for medical research of the Faculty of Medicine Universitas Indonesia (no. No.665/UN2.F1/ETIK/2014). The sample was elected caesarean section derived full term UC, which was obtained after the mother signed an informed consent form. The UC was processed and isolation of MSCs was done by modification of multiple harvest explant method [10].

### Processing of UC

In brief, 5 cm of UC was washed in 0.5% povidone iodine [Betadine] containing phosphate buffered saline pH 7.4 (PBS [Sigma P3813]), followed by washing in PBS. The umbilical arteries and vein were discarded, and the UC tissue was minced into small pieces of 2-5 mm in complete medium to prevent drying. The complete medium was penicillin/ streptomycin (final concentration 100U/mL), amphotericin B (final concentration 2500ng/mL), 1% L-Glutamine (Lonza 17-605C), and 10% platelet concentrate (PC [Indonesian Red Cross]) containing  $\alpha$  minimum essential medium ( $\alpha$ MEM) [GIBCO 12000-022 1]. Then one piece of UC (explant) was put in each well of 24 well plates (growth area 1.9 cm<sup>2</sup> [Biolite]). On each piece, one drop of complete medium was added to prevent drying, and the plates were incubated in 37°C under 5% CO<sub>2</sub>.

### Explant culture

The explant culture was observed everyday. When necessary, one drop of complete medium was added, and when the explant attached to the bottom, 0.5 ml of complete medium was added. The medium was changed every 2-3 days.

### Harvest and passage

Confluent cultures were harvested using TrypLE Select [GIBCO 12563-011], combined and counted. Part of the results of primary cultures were expanded in T25 flasks (seeding around 5000/cm<sup>2</sup>), and the rest was cryopreserved. After harvest, the explants were recultured several times until the explants were detached from the bottom.

The results of passage-1 (P-1) cultures were combined, and counted. Further, part of the P-1 culture was cultured into passage-2 (P-2), and part of P-2 cultures was checked for their differentiation capacity, and the rest was cryopreserved.

### Characterization of the cells

Cryopreserved P-1 derived P-2 cells were characterized for their surface markers (CD90, CD73 and CD34) by flowcytometry, and 10,000 total events were analyzed. Further, the cells were checked for their differentiation capacity into chondrogenic lineage by prolonged culture [11], into adipogenic lineage by adipogenic induction medium (StemPro adipogenesis [GIBCO A10070-01]), and into osteogenic lineage by osteogenic induction medium (StemPro osteogenesis [GIBCO A10072-01]).

### Data collection and analysis

Data collected were cell morphology, property, and characteristics in term of their surface markers and differentiation capacity. Further, number of harvest per well, number of seeding in P-1 and P-2, and number of harvested cells in P-0, P-1 and P-2 were noted. Cumulative harvest number, total seeded cell number in P-1 and P-2, and total harvested cell number in P-0, P-1 and P-2 were calculated. Further, putative total harvested cells in P-1 and P-2 were computed.

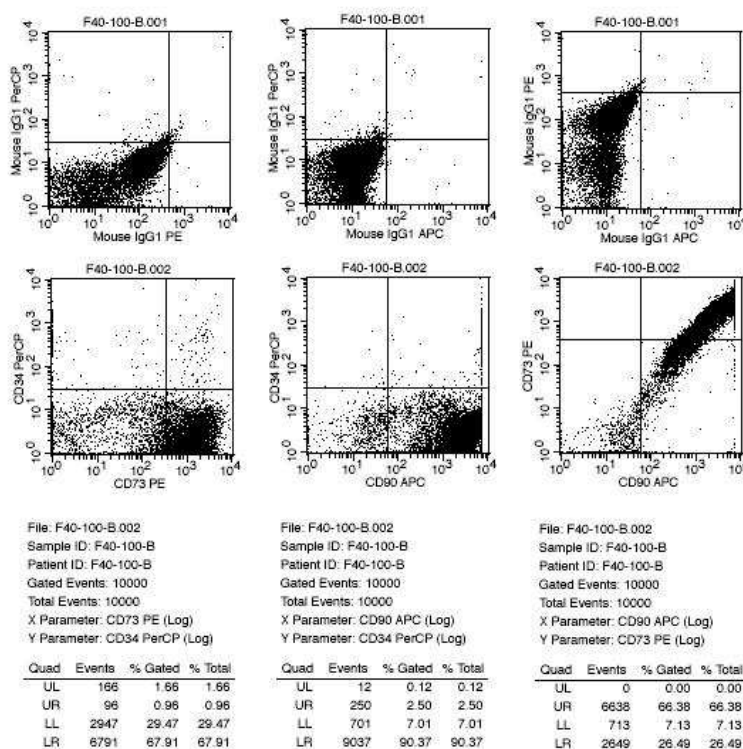
## RESULTS AND DISCUSSION

From 5 cm of UC, we did explant culture in four 24 well plates (total number of wells= 96 wells). The number of harvest per well ranges from 0-5 times i.e. no harvest= 6, once= 20, twice= 34, three times= 27, four times=8, and five times= 1 well(s), respectively. Therefore, we harvested a total of 206 times from the 96 wells, and got a total of 3,595,600 cells from the primary culture.

The cells in P-0, P-1, and P-2 were fibroblastic and plastic adherent. For passage-1, a total of 1,450,560 cells were expanded in twelve T25 flasks, and we got a total of 19,287,600 cells. For passage 2, a total of 871,280 cells were expanded in seven T25 flasks, and we got a total of 18,636,000 cells.

If we use all of the cells from the primary culture result for expansion, we will get a total of 47,809,462 cells in P-1. Further, if we use all of the putative result of P-1 for expansion, we will get a total of 1,022,607,122 cells in P-2, which is equivalent to 20 doses of 50,000,000 cells for patients with 50 kg body weight.

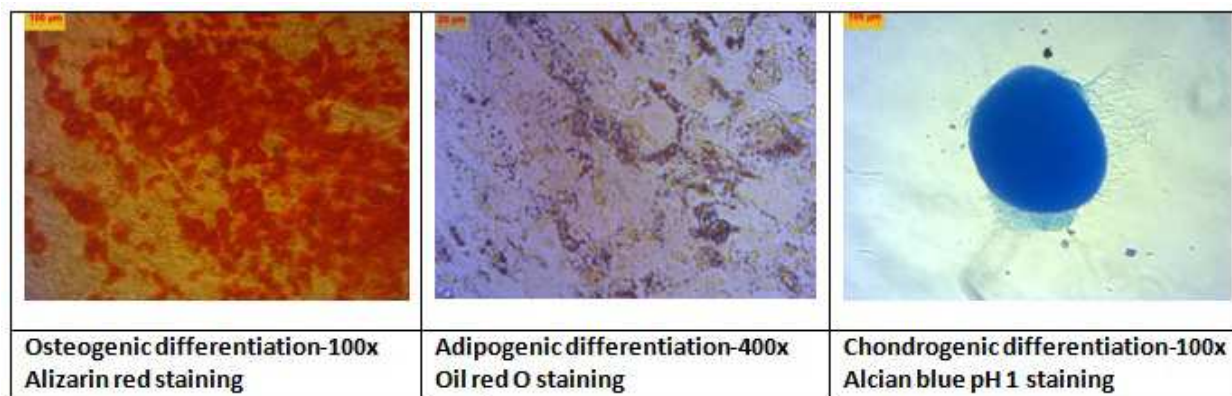
We have developed a simple production method of UC-MSC from 5 cm of UC using xeno-free media that gave enough P-2 cells for around 10 patients (100,000,000 cells per patient). Our method does not require collagenase and hyaluronidase, as in enzymatic method [6, 8]. Moreover, PC is preferred compared to other xeno-material-containing imported supplements such as FBS or FBS derivate containing supplements. Moreover, if we use outdated PC, we can get it free of charge. In our previous studies, we showed that PC contained various growth factors that are needed for cell growth [12], and outdated PC still contained the needed growth factors [13]. Therefore, using outdated PC as supplement can greatly reduce production cost.



**Figure 1. Flowcytometric analysis of P-2 UC-MSCs**  
P-2= passage 2, UC= umbilical cord, MSCs= mesenchymal stem cells

Flowcytometric analysis of P-2 cells showed that CD90, CD73, and CD34 were 92.87%, 67.63%, and 2.62%, respectively (Figure 1). Induction of passage-2 cells showed differentiation capacity into osteogenic, chondrogenic and adipogenic lineage (Figure 2). However, differentiation into adipogenic cells yielded preadipocytes that contain smaller lipid droplets compared to those developed in bone marrow derived MSCs.

Flowcytometric analysis of P-2 cells showed that the surface markers nearly reach International Society for Cell Therapy (ISCT) consensus for mesenchymal stem cells, i.e. CD90 and CD73  $\geq 95\%$ , and CD34  $\leq 2\%$  [14], and further passage might increase CD90 and CD73, and decrease CD34 as was shown in our previous study [15], to meet the consensus criteria of mesenchymal stem cells. Moreover, the presence of CD34 may be preferable as it is found not only on hematopoietic stem cells, but supposed to be present on a proportion of mesenchymal stem cells, and progenitors for various types of cells, including, muscle satellite cells, epithelial and vascular endothelial progenitors [16-18].

**Figure 2. Differentiation capacity of P-2 UC-MSCs**

P-2= passage 2, UC= umbilical cord, MSCs= mesenchymal stem cells

Further, P-2 UC-MSC can differentiate into chondrogenic, osteogenic, and adipogenic lineage, though the lipid droplets in the adipogenic cells were smaller than those in induced bone marrow derived mesenchymal stem cells. This phenomenon was also observed in our previous study [15], and the study of Wagner et al [19], and Kern et al [20] on UC blood MSCs, which showed minimal adipogenic differentiation.

### CONCLUSION

UC-MSCs can be isolated and expanded easily in 10% PC containing alpha MEM, and is suitable to fill the demand of allogeneic MSCs for translational research in regenerative medicine.

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