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Research Article

## Umbilical cord derived mesenchymal stem cell proliferation in various platelet rich plasma and xeno-material containing medium

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### ABSTRACT

Previous studies used fetal bovine serum (FBS) containing medium for umbilical cord derived mesenchymal stem cell culture. Xenoproteins in FBS may be incorporated into the cultured cells and cause immune rejection, when the cells are used in patients. Therefore, the aim of this study was to compare propagation performance of umbilical cord derived stem cells that were cultured in various platelet rich plasma (PRP) containing media and FBS derivate containing commercial medium. This study was an in vitro analytical study on multiple harvest explant culture derived stem cells, which compared the stem cell population doubling time (PDT) of passage 1-3 cultures in human AB PRP containing media (low glucose and high glucose DMEM, and  $\alpha$ MEM) compared to xenoprotein containing commercial medium, MesenCult<sup>®</sup>. Overall, the lowest PDT was achieved by  $\alpha$ MEM-PRP, and PDT in DMEM LG-PRP and  $\alpha$ MEM PRP was comparable to those of MesenCult<sup>®</sup>. Features of cluster of differentiation (CD) expressions in P-1 suggest that the umbilical cord derived stem cells obtained from multiple harvest explant culture were not homogenous for MSCs. However, MSCs tended to become more homogenous with passages, especially for cells that were cultured in MesenCult<sup>®</sup>, followed by those cultured in  $\alpha$ MEM-PRP. In conclusion, the best propagation performance of umbilical cord derived stem cells passage 1-3 cultures was achieved in PRP containing  $\alpha$ MEM.

**Keywords:** Mesenchymal Stem Cell; umbilical cord; Platelet Rich Plasma; population doubling time; flow cytometry

### INTRODUCTION

Umbilical cord is a promising source of stem cells, which acquisition does not pose a risk to the donor as it is a delivery waste. Moreover, the use of umbilical cord derived stem cells do not pose ethical problems. Mesenchymal stem cell (MSC) isolation from umbilical cord tissue is much easier than from umbilical cord blood, (Troyer & Weiss 2008; Weiss et al 2006; Pawitan et al 2013; Pawitan et al. 2014<sup>a</sup>; Secco et al 2008) and isolation by multiple harvest explant method provides more abundant mesenchymal stem cells compared to

conventional explant method. (Pawitan et al 2014<sup>a</sup>)

Many studies cultured umbilical cord derived mesenchymal stem cells in fetal bovine serum (FBS) containing medium. (Yoon et al 2013; Li et al 2013; Maslova et al 2013) However, FBS contains xenoproteins that can be incorporated into the cultured cells, and difficult to be eliminated; thus poses a danger of immune rejection when the cells are used in patients for regenerative medicine. (Spees et al., 2014) To avoid xenoproteins, alternative of FBS, such as human AB platelet rich plasma (PRP) can be used.

Platelet rich plasma is a trombocyte concentrate in human plasma that is available from the Indonesia Red Cross. Thrombin activated PRP was used to culture adipose derived MSCs and was shown to be comparable to fetal calf serum. (Kocaoemer et al 2007) A study reported that 3% PRP supported bone marrow derived mesenchymal stem cell proliferation

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comparable to 2% fetal calf serum (FCS) after passage-4. (Vogel *et al* 2006) Various processing methods to release the growth factors from the platelets in the PRP were reported, and the easiest method is by freezing and thawing in  $-20^{\circ}\text{C}$ . (Pawitan 2012)

A previous study that used PRP from Indonesian Red Cross, used a concentration of 5% and 10% in high glucose Dulbecco's modified Eagle's medium (DMEM-HG) for adipose derived stem cells, which showed that 10% was the optimal concentration, that was better than MesenCult<sup>®</sup> in higher passages. Culture in MesenCult<sup>®</sup> tended to cause more senescent cells, which was shown by increasing cell size with passages. (Suryani *et al* 2013) However, a study showed that culture of rat MSCs in 4.5g/L glucose containing medium like DMEM-HG tended to cause reduced proliferation. (Stolzing *et al* 2006) Therefore, in this study, we compared the population doubling time of multiple harvest explant culture derived umbilical cord MSC (Pawitan *et al* 2014<sup>a</sup>) passage 1-3, in three kinds of media, i.e. low glucose DMEM (DMEM-LG), DMEM-HG, and alpha minimal essential medium ( $\alpha$ MEM) compared to MesenCult<sup>®</sup>.

## MATERIALS AND METHODS

This was an *in vitro* analytical study that was conducted in Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital - Faculty of Medicine, Universitas Indonesia from August 2013 through June 2014. This study got ethical approval from Ethical Committee for medical research of the Faculty of Medicine Universitas Indonesia, ethical clearance No.281/H2.F1/ETIK/2013.

### Samples

Samples were umbilical cords from full term Caesarian section delivered babies due to cephalo pelvic disproportion (CPD), which were obtained from Cipto Mangunkusumo Central Hospital and Bunda Maternity and Child Hospital, Jakarta. Samples taken were from deliveries between 00.00 - 10.00 am.

### Procedures

After the mother signed the informed consent form, 10 cm of umbilical cord was cut and put in a 50 ml transport medium containing tube. Transport medium was 200U Penicillin/ 200 $\mu\text{g}$  Streptomycin (Gibco 15140-122) and 0.5 $\mu\text{g}$  Fungizone (JR Scientific 50701) containing  $\alpha$ MEM (GIBCO 12000-022 1).

Primary cultures were done by multiple harvest explant method, as described previously. (Pawitan *et al* 2014<sup>a</sup>) Passage-1 to passage-3 were cultured in four kinds of media, i.e.  $\alpha$ MEM, DMEM-LG (GIBCO 31600-034) and DMEM-HG (Lonza C15-604 F) that were supplemented by 10% freeze thawed human AB PRP (Indonesia Red Cross) at  $-20^{\circ}\text{C}$ , compared to complete MesenCult<sup>®</sup> medium (Stem Cell technologies basal medium 05401, Stem Cell technologies stimulatory

05402). All media contained a final concentration of 100U Penicillin/100 $\mu\text{g}$  Streptomycin/mL and 0.25  $\mu\text{g}$  Fungizone/mL. A final concentration of 2U/ml heparin (Inviclot) and 1% L-Glutamine (Lonza 17-605C) were added in PRP containing media. For all cultures, seeding was around 20,000 cells in triplicate in twelve well culture plate (Biolite). After seeding, the plates were incubated in  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . Media was changed every 2-3 days until the cultures were 70-80% confluent, and harvested. Cell yields from every culture were counted and population doubling times (PDTs) were calculated. One sample from P-1 and P-3 of each medium was analyzed by flow cytometry for the percentage of CD34, CD73 and CD90. Further, one sample from P-3 of each medium was checked for differentiation capacity into chondrogenic (prolonged culture in 10% PRP containing DMEM-HG), (Pawitan *et al* 2014<sup>b</sup>) adipogenic (Stem Cell Kits SC 006) and osteogenic lineage (Stem Cell Kits SC 006).

### Data analysis

Data collected were PDT and percentage of CD34, CD73 and CD90 of P-1 to P-3, and differentiation data of P-3 in the four kinds of media. The data of PDT were tabulated, and PDT of P-1 to P-3 of the four kinds of media were compared by one way ANOVA, when the data were appropriate, or by Kruskal wallis test, when the data did not follow a normal distribution, or not homogenous. When ANOVA or Kruskal Wallis test showed significant difference in the PDT between the four kinds of media, posthoc analysis using Mann-Whitney test was done.

Percentages of CD34, CD73 and CD90 of P-1 and P-3 in the four kinds of media were presented in Tables, and data of differentiation capacities were presented descriptively.

## RESULTS

We got five samples of umbilical cords. Mean PDT of P1-P3 in the four media can be seen in Table 1, and posthoc analysis of the difference between media can be seen in Table 2. Longest PDT occurred in DMEM HG-PRP.

Results of flow cytometric analysis of P-1 and P-3 in the four kinds of media can be seen in Figure 1-3. In all media, P-1 showed CD90 percentage of more than 70%, which was increased to 80% in P-3, except for DMEM HG-PRP (50%). Percentage of CD73 was rather low for MSC, except that was cultured in a commercial medium special for MSCs (MesenCult<sup>®</sup>). Further, P-3 cells from the four kinds of media showed differentiation capacity into adipogenic, chondrogenic and osteogenic lineage. However, the lipid droplets in adipogenic differentiation in this study were smaller compared to those from lipoaspirate derived MSCs in our previous study. (Suryani *et al* 2013; Pawitan *et al* 2014<sup>b</sup>).

**Table 1: Mean of PDT in P1 to P3 in various media**

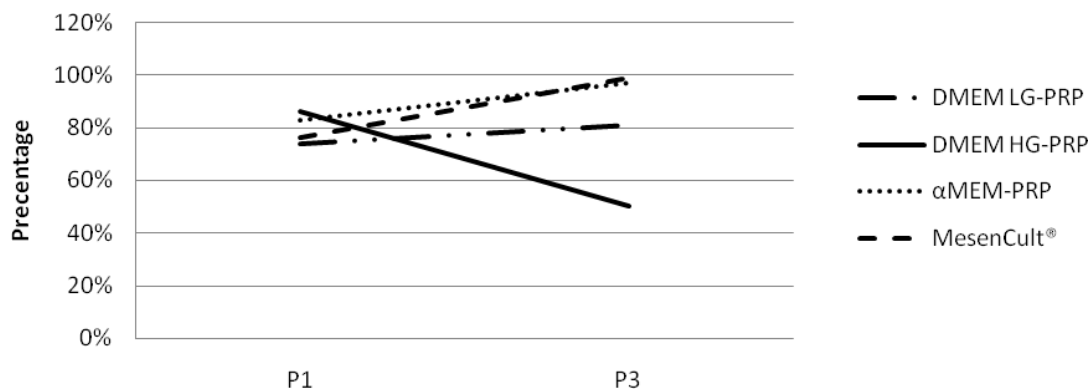
Passage	DMEM HG-PRP	DMEM LG-PRP	αMEM PRP	Mesencult®
P-1	3.5±1.7	2.3±1.0	2.6±0.8	3.0±1.6
P-2	3.8±2.0	2.4±0.6	2.0±0.8	2.4±0.7
P-3	2.3±1.2	2.4±1.6	2.3±1.7	2.3±1.4
Mean	3.2±1.6	2.4±1.1	2.3±1.1	2.6±1.2

PDT = population doubling time, DMEM= Dulbecco’s modified Eagle’s medium, HG= high glucose, LG= low glucose, PRP= platelet rich plasma 10%, αMEM= α minimal essential medium, P-1= passage 1, P-2= passage 2, P-3= passage 3.

**Table 2: Posthoc analysis of PDT in P1-P3 in various media**

Medium	DMEM HG-PRP	DMEM LG-PRP	αMEM PRP	MesenCult®
P1 DMEM HG -PRP	--	0.064	0.268	0.407
P1 DMEM LG-PRP	0.064	--	0.410	0.307
P1 αMEM PRP	0.268	0.410	--	0.810
P1 MesenCult®	0,407	0.307	0.810	--
P2 DMEM HG- PRP	--	0.060	0.000*	0.029*
P2 DMEM LG-PRP	0.060	--	0.045*	0.717
P2 αMEM PRP	0.000*	0.045*	--	0.110
P2 MesenCult®	0.029*	0.717	0.110	--
P3 DMEM HG-PRP	--	0.918	0.533	0.932
P3 DMEM LG-PRP	0.918	--	0.623	0.988
P3 αMEM PRP	0.533	0.632	--	0.621
P3 MesenCult®	0.932	0.988	0.621	--

PDT = population doubling time, DMEM= Dulbecco’s modified Eagle’s medium, HG= high glucose, LG= low glucose, PRP= platelet rich plasma 10%, αMEM= α minimal essential medium, P-1= passage 1, P-2= passage 2, P-3= passage 3.



**Figure 1: Percentage of CD90 in P-1 and P-3 in various media**

**Discussions**

Our previous study showed that adipose tissue derived mesenchymal stem cells performed well in 10% PRP supplemented DMEM-HG in term of PDT, but MesenCult® tend to cause senescence that was showed by increase in cell size with passaging. (Suryani et al 2013) DMEM-HG was used in several studies, (Suryani et al 2013; Ayatollahi et al 2012; Pawitan et al 2011) and was shown to cause lost of proliferation capacity between P-5 and P-7 in bone marrow MSCs, (Ayatollahi et al 2012) and spontaneous differentiation in a small percentage of the cells in adipose derived stem cells. (Pawitan et al 2011) Moreover, a study showed that culture in DMEM-HG tended to cause senescence. (Stolzinger et al 2006) Therefore, we compared 10% PRP

supplemented DMEM-HG with DMEM-LG that contains less glucose, and αMEM. αMEM contains less essential amino acids and vitamins compared to DMEM. (Sigma-Aldrich<sup>a</sup>; Sigma-Aldrich<sup>b</sup>) Further, we also compared the three PRP supplemented medium with MesenCult® that should be supplemented with an FBS derivate containing supplement. MesenCult® is a commercial medium that is special for MSC growth.

**Population doubling time**

Overall, the lowest PDT was achieved by αMEM-PRP, and PDT in DMEM LG-PRP and αMEM PRP was comparable to those of MesenCult®. Further, P-1 and P-2 cells that were cultured in PRP supplemented DMEM HG-PRP showed the highest PDT compared to

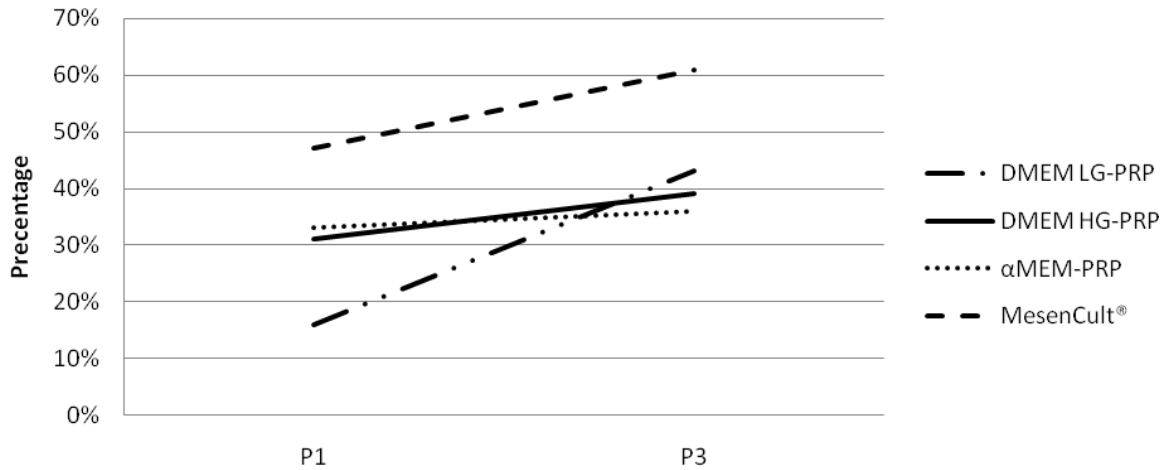


Figure 2: Percentage of CD73 in P-1 and P-3 in various media

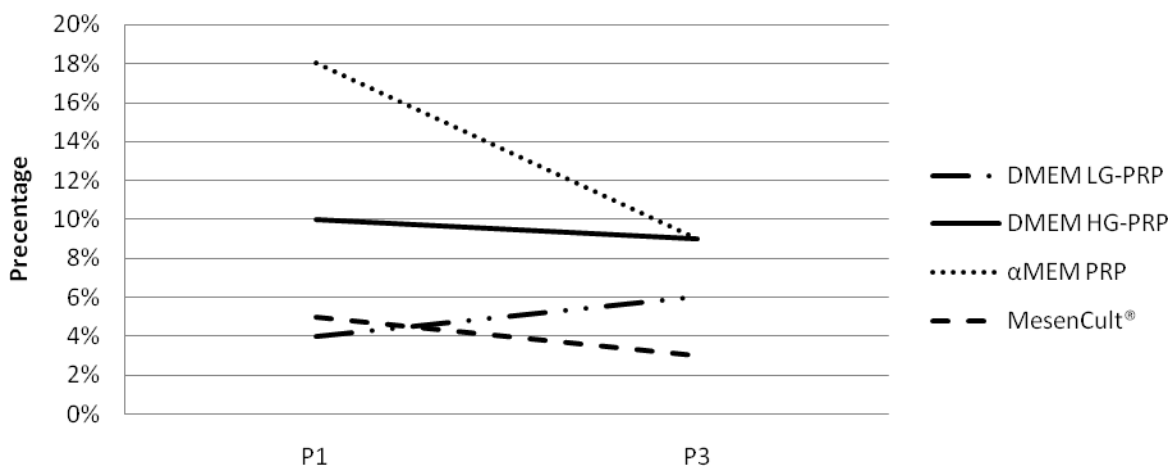


Figure 3: Percentage of CD34 in P-1 and P-3 in various media

those in DMEM LG-PRP and αMEM-PRP (Table 1), though the PDT difference in P-1 was not significant statistically (Table 2). High glucose was shown to cause senescence, which may interfere with proliferation capacity, thus reduced proliferation, (Stolzing et al 2006) which was corroborated by our study. Moreover, high glucose increased apoptosis. (Saki et al 2013) However, a study showed that high glucose did not impair proliferation in MSC, and favor differentiation into osteogenic lineage. (Li et al 2007) In addition, another study showed that αMEM–10% FBS tended to cause more senescence and spontaneous differentiation into adipocytes compared to DMEM LG-10% FBS, (Lee et al 2010) which is the opposite to our study that showed the lowest PDT occurred in αMEM-PRP. This difference might be due to different supplement, which was PRP in our study, while FBS was used in Li et al and Lee et al study. (Li et al 2007; Lee et al 2010)

Moreover, the cells that were cultured came from umbilical cord explant culture, which might contain myriads of stem cells and progenitors in various stages of differentiation. Umbilical cord contains various kinds of stem cells from various compartments, e.g.

intervascular Wharton’s jelly MSCs, epithelial stem cells, cord lining/sub amnion (CLEC-muc) stem cells, (Reza et al 2011) and perivascular stem cells. (Troyer & Weiss 2008; Jeschke et al 2011) The various kinds of media used in this study might favor a certain kind of those cells, while causing differentiation or senescence of other cells.

**Flow cytometric analysis and differentiation capacity**

Features of CD expressions in P-1 suggest that the umbilical cord derived stem cells obtained from multiple harvest explant culture were not homogenous for MSCs. However, MSCs tended to become more homogenous with passages, especially for cells that were cultured in MesenCult®, which is a special commercial medium for the expansion of MSCs, followed by those cultured in αMEM-PRP. This facts were showed by the increase in CD 90 and CD 73, and decrease in CD 34 in P-3 (Figure 1-3).

Percentage of CD90 in P-3 from αMEM-PRP and MesenCult® were both above 90% that showed a typical feature of MSCs. However, the percentage of CD73 even in P-3 in MesenCult® did not meet the consensus criteria of MSCs that was published by Dominici et al (2006), which was a criteria intended for

bone marrow derived MSCs. (Dominici et al 2006) Lower level of CD73 compared to the consensus level was also found by Hao et al (2013). (Hao et al 2013) In this study, percentages of CD34 were variable and was high in  $\alpha$ MEM-PRP, which was around 18%. However, the percentage tended to decrease with passaging, except culture in DMEM LG-PRP, which favored for CD34 (Figure 3). The presence of CD34 might come from umbilical cord blood (Eggermann et al 2003) that was still present inside the capillaries in umbilical cord tissue. In addition, CD34 was also found in early passages of lipoaspirate derived MSCs, which derived from the perivascular fraction. (Mitchel et al 2006) Therefore, the criteria of MSCs might need a new consensus, which can accommodate the various tissue derived MSCs that might show subtle differences in their CD expressions. Moreover, culture medium might also interfere with cell phenotype, (Pombinho et al 2004) and thus the expression of CD molecules.

Overall, the medium that most favor the growth of MSCs according to the consensus (Dominici et al 2006) was MesenCult<sup>®</sup>, followed by  $\alpha$ MEM-PRP. However, recent opinions suggested that the presence of CD34 might be favorable for regenerative medicine, as CD34 might represent various kinds of progenitors, (Sidney et al 2014) including endothelial progenitors, (Eggermann et al 2003; Peichev et al 2000) which is important for angiogenesis that is needed in tissue repair.

In this study, differentiation analysis showed that P-3 in the four kinds of media showed differentiation capacity into adipogenic, chondrogenic, and osteogenic lineage, which revealed that the stem cells that were obtained from multiple harvest explant method were mesenchymal stem cells, though the lipid droplets were smaller than those of adipose derived MSCs. (Suryani et al 2013; Pawitan et al 2014b) This finding was in line with Karahuseyinoglu et al's result, which found that the umbilical cord MSCs were only able to be differentiated into immature adipocytes. (Karahuseyinoglu et al 2007)

Therefore, based on some findings that umbilical cord derived MSCs do not strictly match the consensus CD criteria, we can consider the stem cells that were grown in PRP containing  $\alpha$ MEM as MSCs that are suitable for regenerative medicine.

## CONCLUSION

The best propagation performance of passage 1-3 cultures was achieved by PRP containing  $\alpha$ MEM.

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