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International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.4, pp 1202-1208, Aug-Sept 2014

Umbilical Cord Derived Stem Cell Culture: Multiple-Harvest Explant Method

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Abstract: Published methods of umbilical cord explant culture require removing the explant after the MSCs are confluent. However, we observed that the explants were still attached after the MSCs were detached using TrypLE Select. Therefore, the objective of this study was to do explant cultures in xeno-free media, and re-culture the explants after the first harvest to test whether the explant could grow further to yield more stem cells. In this study, umbilical cord explant culture were done in 10% platelet rich plasma (PRP) containing alpha minimal essential medium (αMEM) or Dulbecco's modified Eagle's medium (DMEM), or 10% human AB or cord blood serum (CBS) containing αMEM. Three explants were cultured in each well of a twelve well plate in triplicate. The cells were harvested using TrypLE Select, and cell yield was counted after cell growth achieved 80-90% confluence. The remaining attached explants were re-cultured in the respective media for several times after harvesting, until the explants were detached or until there was no more cell growth from the explants. Cell counts for every harvest were noted. Cumulative cell yield from each well for each medium was computed and tabulated. Our results showed that the time needed for cell growth was variable between explants. Therefore, in one well, the three explants might grow with different speed, and in many instances, harvests were done before optimal growth in all explants occurred. The maximal number of harvest was four times, which occurred in 10% human AB serum supplemented αMEM (two times) followed by two harvest in 10% autologous CBS supplemented αMEM, and yielded a cumulative amount of 144,000 viable cells in 37 days. In conclusion, we have developed a multi-harvest explant method to culture umbilical cord stem cells in various xeno-free media that yield far more numerous viable cells compared to standard explant method.
Key words: umbilical cord, explant culture, multiple harvests, stem cell, thrombocyte concentrate.

Introduction

Umbilical cord is a rich source of mesenchymal stem cells (MSCs).[1] Umbilical cord derived MSCs are regarded less immunogenic and have immunosuppressive properties as bone marrow-derived MSCs.[2] These MSCs can also be stored in cell banks, and therefore are readily available when the patients need them. Therefore, they become a good candidate to be used in regenerative medicine as allogeneic MSCs.

Most of umbilical cord stem cell isolation methods used fetal bovine serum (FBS) as supplements.[3-6] However, to be used in patients, the MSCs should be processed and cultured in xeno-free media, as xeno or animal-derived proteins can be incorporated into the stem cells, and difficult to be eliminated. [7] Recently, some FBS substitutes were developed, such as autologous[1] or allogeneic[8] human serum and thrombocyte concentrate (TC) or platelet rich plasma (PRP). Thrombocyte concentrate have been successfully used to culture bone marrow[9] and adipose tissue[10,11] derived MSCs. Therefore, our study explored the use of human AB thrombocyte concentrates from Indonesian Red Cross, which were tested and proven negative for bacterial and viral infections, compared to commercial human AB serum, and umbilical cord blood serum.

The easiest way to isolate the MSCs from umbilical cord is by explant method.[1,3,8] The published methods of umbilical cord explant culture require removing the explant after the MSCs are confluent. [1,3,8] However, we observed that the explants were still attached after the MSCs were detached using TrypLE Select. Therefore, in this study we attempted to further culture the explants, and to test whether the explant could grow further to yield more MSCs.

Materials and Methods

This study was an experimental descriptive study, which was done in Culture Laboratory of Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital-Faculty of Medicine Universitas Indonesia, in August - November 2013. Ethical clearance was obtained from the Ethical Committee, Faculty of Medicine, Universitas Indonesia. Full term normal umbilical cord was obtained from Caesarean section, in Department of Obstetrics and Gynecology, Cipto Mangunkusumo Hospital, and Bunda Maternal and Child Hospital, Jakarta, after the mother signed the informed consent.

Procedure

Ten cm of umbilical cord was collected in 50 mL transport medium, which contained alpha minimal essential medium (αMEM [GIBCO 12000-022 1]), penicillin/streptomycin (final concentration 300U/mL [Gibco 15140-122]) and amphotericin B (final concentration 7500ng/mL [JR Scientific 50701]), and processed in less than 8 hours after collection.

The umbilical cord was dissected and washed briefly in 0.5% povidone iodine (betadine) containing phosphate buffered saline pH 7.4 (PBS [Sigma P3813]), followed by washing in PBS to remove blood and betadine. Further, the umbilical arteries and vein were dissected and discarded, and the umbilical cord was minced in complete medium. We used alpha MEM and Dulbecco's modified Eagle's medium (DMEM,[GIBCO 31600-034]). The complete media contained penicillin/ streptomycin (final concentration 100U/mL), amphotericin B (final concentration 2500ng/mL), 1% L-Glutamine (Lonza 17-605C), and 10% TC (Indonesian Red Cross). For αMEM, cultures were also supplemented by 10% human AB serum (Gibco 34005-100), 10% autologous or allogeneic umbilical cord blood serum. All together, there were four kinds of complete media.

Three explants (diameter 2-5 mm) with Wharton's jelly facing downward were placed in each well of a 12 well plate (growth area 3.8 cm2 [Biolite]), and several drops of the respective complete medium were added. For all media, the cultures were done in triplicate. Further, the plate was incubated in 37°C, 5% CO_2. The cultures were observed daily to detect cell growth, or contamination. When contamination occurred, the contaminated wells were eliminated. When the explant attached to the plastic, 200 -500 µl of respective medium was added. Medium change was done every 2-3 days, by removing half of the medium, and adding half fresh respective medium.

When the cells grew out from the explants and became 90% confluent, they were harvested using TrypLE Select (GIBCO 12563-011), and viable/non viable cell yield was counted using dye exclusion method. When the explant was still attached, new respective medium was added, and the plate was again incubated in 37°C, 5% CO_2. The second and successive cultures were observed daily to detect cell growth, and when the cells attained 90% confluence, they were harvested. Therefore, one explant could be harvested several times.

Data collection

Data collected were day of harvest (counted from the beginning of initial and successive primary cultures), type of culture (initial or successive primary cultures), the medium, and number of viable cells harvested. The data were presented in a Table.

Results and Discussion

We got two samples, the first (S1) was processed three hours after collection, while the second (S2) was processed 7.5 hours after collection. For sample 1, autologous umbilical cord blood serum (CBS) was available, but used after culture in human AB serum, as for the first culture, the CBS was not ready. For sample 2, autologous CBS was not available, and we used allogeneic CBS that was prepared before. For this study, around two cm of the ten cm was sufficient to generate all the cultures in triplicate.

The time needed for cell growth was variable between explants. Therefore, in one well, the three explants might grow with different speed, and in many instances, harvests were done before optimal growth in all explants occurred. The type of harvest, well location, day of harvest, viable cell yield, and number of sprouting explants in the four kinds of media for the two samples can be seen in Table-1.

Table 1. Type and day of harvest, viable cell yield and number of sprouting explants in various media

Media /sample	Location/type of harvest	Day of harvest	Viable: non viable cell yield	Number of sprouting explant
DMEM-TC/S1	Well-1/initial	Day-19	9,600: 0	2 explants
	Well-1/second	Day-12	25,200: 3,600	2explants
	Well-1/third	Day-7	17,600: 3,200	1explant
	Well-2/initial	Day 12	39,600: 2,400	1 explant
	Well-2/second	Day-6	62,400: 4,800	2 explants
	Well-2/third	Day-6	12,000: 4,800	1 explant
	Well-3/initial	Day-17	13,200: 3,600	3 explants
	Well-3/second	Day-17	8,400: 2,400	1 explant
DMEM-TC/S2	Well-1/initial	Day-17	12,000:0	2 explants
	Well-1/second	Day-7	7,200:0	1 explant
	Well-2/initial	Day-11	25,600: 6,400	3 explants
	Well-2/second	Day-5	19,200: 4,800	1 explant
	Well-2/third	Day-8	36,000:0	1 explant
	Well-3/initial	Day-17	57,600:3,200	3 explants
	Well-3/second	Day-8	19,200:6,000	1 explant
αMEM-TC/S1	Well-1/initial	Day-36	153,600: 9.600	3 explants
	Well-2/initial	Day-30	31,200: 1,200	1 explant
	Well-3/initial	Day-19	14,400: 9,600	1 explant
	Well-3/second	Day-5	19,200; 3,600	1 explant
	Well-3/third	Day-8	8,400: 1,200	2 explants
αMEM-TC/S2	Well-1/initial	Day-11	28,800: 0	3 explants
	Well-1/second	Day -5	76,800:4,800	3 explants
	Well-1/third	Day-4	8,400: 1,200	1 explant
	Well-2/initial	Day-11	110,000: 0	3 explants
	Well-2/second	Day-5	33,600: 4,800	1 explant
	Well-3/initial	Day-12	20,400: 0	2 explants
	Well-3/second	Day-7	12,000: 2,400	2 explants
αMEM-hu AB/S1	Well-1/initial	Day 19	13,800: 3,600	3 explants
	Well-1/second	Day 6	10,800: 1,200	2 explants
	Well-2/initial	Day-24	32,400: 6,000	3 explants
	Well-3/initial	Day 19	9,600:0	3 explants
	Well-3/second	Day-6	27,600: 3,600	3 explants
αMEM-hu AB/S2	Well-1/initial	Day-10	50,400: 2,400	3 explants

	Well-1/second	Day-6	52,800: 2,400	3 explants
	Well-1/third	Day-8	64,800: 7,200	2 explants
	Well-2/initial	Day-11	36,000: 3,600	3 explants
	Well-2/second	Day-5	48,200: 2,400	3 explants
	Well-2/third	Day-5	22,800:0	2 explants
	Well-3/initial	Day-11	54,000: 0	1 explant
	Well-3/second	Day-8	76,800:13,200	3 explants
	Well-3/third	Day-5	28,800:7,200	2 explants
αMEM-auto CBS, after hu AB/S1	Well-1/third	day-7	42,000: 2,800	3 explants
	Well-2/second	Day-9	52,000: 4,000	3 explants
	Well-3/third	Day-9	86,400: 7,200	3explants
	Well-3/fourth	Day-7	20,400: 4,800	1 explant
αMEM-CBS-S2	Well-1/initial	Day-10	52,600: 6.000	3 explants
	Well-1/second	Day-8	35,200:0	1 explants
	Well-1/third	Day-7	48,000: 2,400	3 explants
	Well-2/initial	Day-10	15,600: 0	2 explants
	Well-2/second	Day-8	57,200: 4,400	3 explants
	Well-2/third	Day-7	42,000: 11,200	1 explant
	Well-3/initial	Day-10	14,400: 6,000	3 explants
	Well-3-second	Day-8	66,000: 4,400	3 explants

DMEM= Dulbecco's modified Eagle's medium, MEM= minimal essential medium, TC= thrombocyte concentrate 10%, hu AB= human AB serum 10%, CBS= umbilical cord blood serum 10%, S1= sample 1, S2= sample 2.

Further, the number of harvest, cumulative time needed and viable cell yield for the various media can be seen in Table 2. In this study, the maximal number of harvests was four times, which occurred in αMEM supplemented by 10% human AB serum (two times) followed by two harvest in αMEM supplemented by 10% autologous CBS, and yielded a cumulative amount of 144,000 viable cells in 37 days (Table 2).

Table 2. Cumulative viable cell yield in various media

Media/sample/ well	Number of harvest	Cumulative time needed	Cumulative viable cell yield	
			Viable cell number	Mean (SD)
DMEM-TC/S1/w-1	3	35 days	52,400	62,667 (47,048)
DMEM-TC/S1/w-2	3	21 days	114,000	
DMEM-TC/S1/w-3	2	32 days	21,600	
DMEM-TC/S2-w-1	2	22 days	19,200	58,933 (34,468)
DMEM-TC/S2-w-2	3	21 days	80,800	
DMEM-TC/S2-w-3	2	23 days	76,800	
αMEM-TC/S1-w-1	1	35 days	153,600	75,600 (67,765)
αMEM-TC/S1-w-2	1	29 days	31,200	
αMEM-TC/S1-w-3	3	29 days	42,000	
αMEM-TC/S2-w-1	3	17 days	114,000	96,667 (57,591)
αMEM-TC/S2-w-2	2	14 days	143,600	
αMEM-TC/S2-w-3	2	17 days	32,400	
αMEM-hu AB/S1+ αMEM-auto CBS-w-1	2 + 1= 3	23 + 6= 29 days	24,600 + 42,000= 66,600	98,333 (40,538)
αMEM-hu AB/S1+ αMEM-auto CBS-w-2	1 + 1= 2	23 + 8= 31 days	32,400 + 52,000= 84,400	
αMEM-hu AB/S1+ αMEM-auto CBS-w-3	2 + 2= 4	23 + 14= 37 days	37,200 +106,800= 144,000	
αMEM-hu AB/S2-w-1	3	21 days	168,000	144,867 (33,061)
αMEM-hu AB/S2-w-2	3	18 days	107,000	

αMEM-hu AB/S2-w-3	3	21 days	159,600	
αMEM-CBS-S2-w-1	3	22 days	135,800	110,333 (27,969)
αMEM-CBS-S2-w-2	3	22 days	114,800	
αMEM-CBS-S2-w-3	2	16 days	80,400	

DMEM= Dulbecco's modified Eagle's medium, MEM= minimal essential medium, TC= thrombocyte concentrate 10%, hu AB= human AB serum 10%, CBS= umbilical cord blood serum 10%, S1= sample 1, S2= sample 2, w-1= well-1, w-2= well-2, w-3= well-3

In this study, the shortest time needed to reach 90% confluence for initial culture was 9 days (on day-10, Table 1), and the time needed to reach 90% confluence seemed to be influenced by the type of complete medium and the sample itself. Overall, sample 1 needed a longer time to achieve 90% confluence compared to sample 2 (Table 1). Overall, initial culture needed longer time to reach 90% confluence compared to second or more cultures, and the longest time needed for initial culture was 35 days (day-36) for αMEM supplemented with 10% TC. Moreover, second or more cultures tended to yield more viable cells, before they became exhausted that was indicated by reduced viable cell yield. Therefore, this multiple harvest explant method yielded more viable cells in shorter time, compared to the usual explant method.

For initial culture, we got a minimum of 3,200 and a maximum of 51,200 viable cells per explant from αMEM supplemented with 10% human AB serum and TC, respectively. The minimum amount of viable cell yield was due to localized out growth (sprouting) of cells in a small area of one explant that reached 90% confluence (Figure 1), while the other explants had not reach 90% confluence. Moreover, in some wells, not all explants gave cell out growth at the same time. In certain wells, only one explant grew in initial culture, followed by more explants that grew in further cultures (Table 1).

A study by de Bruyn et al (2011) obtained a mean of 1.4×10^8 cells at the second and $>7\times10^9$ cells at the third passage respectively, from 5-10 cm of umbilical cord, [12] thus, one passage increased the number 50 times. In our study, the least viable cell yield was in DMEM supplemented with 10% TC, which was 58,933viable cells per well. If we calculated the viable cell yield from 10 cm of umbilical cord, we would have around $3.5\text{x}10^6$ viable cells (58,933 x 12 wells x 10 cm/2 cm) from the primary culture (P0). Therefore, first, second, and third passage would yield $1.8\text{x}10^8$, $9\text{x}10^9$, and $45\text{x}10^{10}$ viable cells respectively, which was far more numerous than the study of de Bruyn et al.[12]

A study by Hatlapatka et al (2011) compared various concentration of human serum as supplement to αMEM for umbilical cord explant culture and found that the best human serum concentration was 10% and showed that the cells that grew were MSCs.[3] The cells from our explant cultures were fibroblastic and plastic adherent. Therefore, they were highly possible to be MSCs, as one of the media was 10% human AB serum containing αMEM. However, we did not check the surface markers of the cells, which was the limitation of our study.

Conclusion

We have developed a multi-harvest explant method to culture umbilical cord stem cells in various xeno-free media that yield far more numerous viable cells compared to standard explant method.

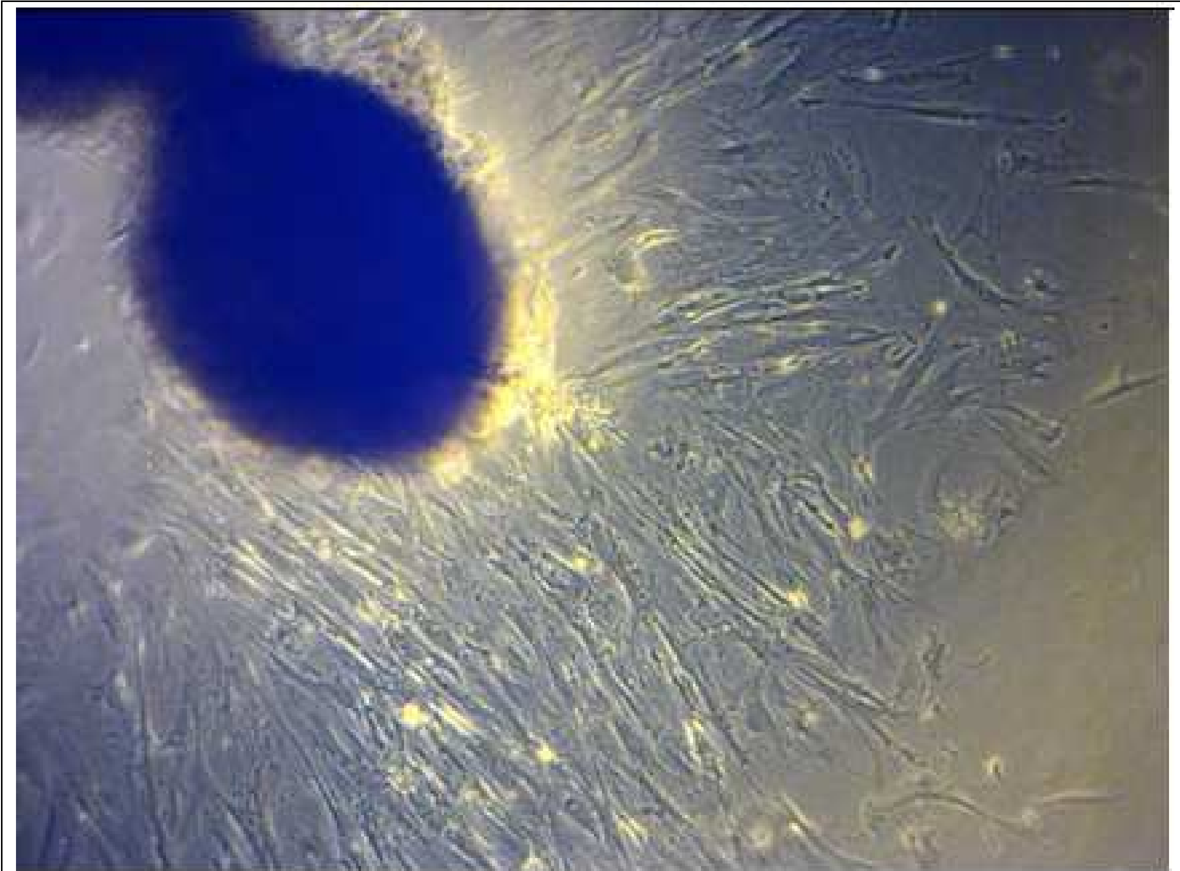

Figure 1. Localized sprouting of explant culture

Acknowledgements

This study was funded by the grant from Directorate of Research and Community Service of Universitas Indonesia 2013, contract no. 0947/H2.R12/HKP.05.00/2013. We are greatly indebted to the residents, doctors, and paramedical staff of Department of Obstetrics and Gynecology, Cipto Mangunkusumo Hospital and Bunda Maternal and Child Hospital, Jakarta, who helped in taking the samples, and the patients, who donated the umbilical cord samples.

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