International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.7, No.2, pp 354-359, 2014-2015

Simple Method to Isolate Mesenchymal Stem Cells from Bone Marrow using Xeno-Free Material: A Preliminary Study

Jeanne Adiwinata Pawitan[1, 2]*, Lauda Feroniasanti[1], Tera Kispa[1], Ismail Hadisoebroto Dilogo[1, 3], Iqbal Fasha[1], Trie Kurniawati[1], Isabella Kurnia Liem[1, 4]

[1]Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital - Faculty of Medicine Universitas Indonesia, CMU 2 Building, 5th floor, Jl. Diponegoro 71, Jakarta, Indonesia
[2] Department of Histology, Faculty of Medicine Universitas Indonesia, Jl. Salemba 6, Jakarta, Indonesia
[3]Department of Orthopaedic and Traumatology, Faculty of Medicine Universitas Indonesia, Jl. Salemba 6, Jakarta, Indonesia
[4]Department of Anatomy, Faculty of Medicine Universitas Indonesia, Jl. Salemba 6, Jakarta, Indonesia

Abstract: Attempts to look for FBS substitute showed that platelet rich plasma (PRP) was able to replace fetal bovine serum (FBS), and in some studies even showed better performance than FBS. However, most studies that compared FBS to xeno-free materials such as platelet rich plasma, allogeneic human AB serum, or autologous plasma to culture bone marrow derived stem cells did not conduct the study on primary cultures, as they used passaged cells. Therefore, in this study we used PRP supplemented medium in primary cultures to isolate MSCs from bone marrow without mononuclear cell enrichment step. Processing of bone marrow was done by simple centrifugation at 400g to separate the pellet from supernatant, followed by centrifugation of supernatant to get the remaining stem cells. Both first and second pellets were cultured in 10% PRP containing alpha minimum essential medium (αMEM). The time needed for the cells to attach in the supernatant derived primary culture, time to become confluent and harvested, cell yields in primary culture harvest (pellet and supernatant) were noted. After second centrifugation, supernatant yielded a pellet, and both pellets from first and second centrifugation contained mesenchymal stem cells (MSCs) that can be isolated due to their plastic adherent property. Supernatant derived pellet culture became confluent faster compared to those from first centrifugation. In conclusion: PRP supplemented α MEM can be used to isolate MSCs from bone marrow using simple centrifugation method.
Keywords: Bone marrow; Mesenchymal stem cell; Platelet rich plasma; Xeno-free.

Introduction

Mesenchymal stem cells (MSCs) have been used in various clinical trials,[1-5] and showed promising results in *in vitro* studies and animal models for various kinds of degenerative diseases.[3, 6-8] In classical media composition and most previous studies, MSCs were isolated using fetal bovine serum (FBS) supplementation.[9] However, FBS contains xeno-material and a study showed that FBS induced humoral response in rats;[10] thus the use of FBS in culture medium might pose rejection problems in patients, which might be severe as in anaphylactic shock. Therefore, it is very important to avoid the use of xeno-material in the isolation, propagation, and processing of MSCs for patients' use.

Attempts to look for FBS substitute showed that platelet rich plasma (PRP) was able to replace FBS, and in some studies even showed better performance than FBS.[11] Platelet rich plasma can be purchased from blood banks, and is guaranteed to be free from infectious disease causing organisms; thus is safe for patients. The use of PRP in culture needs processing to release growth factors from the platelets. However, various studies used various PRP processing, i.e. thrombin, thrombin-CaCl2 activation, or freeze thaw cycles using various freezing temperatures and various cycle numbers.[11] A study on freeze thaw cycles using -20^{o}C as freezing temperature that compared one to three cycles showed that there was no significant difference in growth factor levels between one, two, and three cycles.[12]

Most studies that compared FBS to xeno-free materials such as PRP, allogeneic human AB serum, or autologous plasma used FBS for isolation (primary cultures) of bone marrow (BM) and adipose tissue (AT) derived MSCs, and did comparisons on passaged cells.[13-16] Specifically for BM derived MSCs, most studies used mononuclear cell enrichment by gradient centrifugation before primary culture to isolate MSCs.[9, 13, 14] Recently, a simple method was described without mononuclear cell enrichment, which directly cultured rabbit BM derived pellet in FBS containing medium after simple centrifugation at 400g. [17]

Therefore in this study, we combined the method to isolate MSCs from bone marrow without mononuclear cell enrichment step and the use of 10% PRP containing medium in primary culture to isolate the MSCs.

Materials and Methods

This was an *in vitro* study that was conducted in Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Central Hospital - Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia, from June through July 2014. Ethical approval for this study was obtained from the Ethic Committee for Medical Research, Faculty of Medicine Universitas Indonesia, ethical clearance no 165/H2.F1/ETIK/2014.

Samples

Bone marrow samples (10-12 ml) were taken from donor's iliac crest or medullary femoral bone, after they signed the informed consent form. The samples were transported in heparin containing syringe and were processed in one hour.

Procedures

The samples were transferred into two 15 ml conical tubes, and a same amount of complete medium was added. The complete medium consisted of a final concentration of 100U Penicillin/100µg Streptomycin/ml (Gibco 15140-122) , 0.25 µg Fungizone/ml (JR Scientific 50701), 10U/ml heparin (Inviclot), 1% Glutamax (GIBCO A12860-01), 10% PRP (Indonesian Red Cross), and alpha minimum essential medium (αMEM [GIBCO 12000-022 1]). The samples were centrifuged at 400g (Hettich EBA20) for 10 minutes. The supernatant were recovered and pellets were transferred into a T25 flask. A same volume of complete medium was added to the pellets. The flask was incubated in 37^{o}C and 5% CO2.

The supernatant was centrifuge at 400 g for 10 minutes, and the pellet were re-suspended in 1 ml complete medium and cultured in a well of a 12 well plate, in 37^{o}C and 5% CO2. The well was observed daily to look for attached fibroblastic cells. When enough fibroblastic cells were attach, both the well and flask were washed in 1 ml and 5 ml phosphate buffered saline pH 7.4 (Sigma P3813), respectively several times until they were free from red blood cells. Further, 1 ml and 5 ml of fresh complete medium was added to the well and flask respectively. Both plate and flask were observed daily, and every 2-3 days, medium changes were done, until one of the clones was 90% confluent, and ready for harvesting.

The cells were detached using TrypLE Select (Gibco 12563-011), and viable cell yields were counted using trypan blue dye exclusion method, and noted. Sample-1 pellet culture was passaged in three T25 flasks, and part of passage-1 (P-1) MSCs was induced to differentiate into chondrocyte (prolonged culture method)[18], osteocyte (StemPro osteogenesis [GIBCO A10072-01]), and adipocyte (StemPro adipogenesis [GIBCO A10070-01]).

Data collection and analysis

Data collected were time needed for the cells to attach in the supernatant derived primary culture, time to become confluent and harvested. Cell yields in primary culture harvest (pellet and supernatant) were noted. The data were presented as tables.

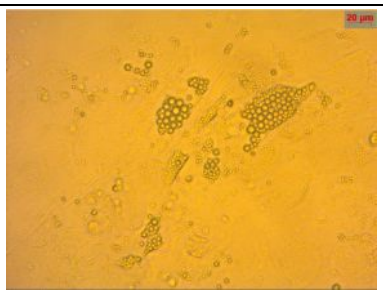
Results And Discussion

We got three samples; two samples were taken from iliac crest and one from medullary femur (femoral fracture). Samples that were taken from iliac crest contained few fat cells, but the sample from medullary femur contained a lot of fat cells, which were difficult to wash. Supernatant derived primary cultures were contaminated by osteoclast-like cells, especially sample-1, but the contaminants were still attached after five minutes in TrypLE Select. Therefore, minimizing the time in TrypLE Select might avoid further contamination.

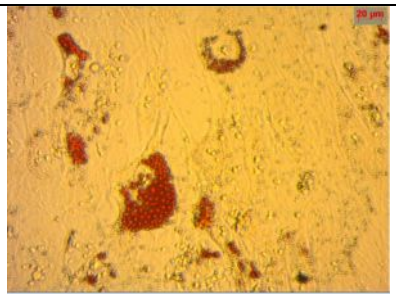
The first time fibroblastic cells appeared in supernatant derived primary culture, time to become confluent, and cell yields in pellet and supernatant derived primary culture can be seen in Table 1. Yield of P-1 was 4,500,000 MSCs. P-1 induced cells that differentiated into chondrocytes, osteocytes and adipocytes can be seen in Figure-1.

Table 1. Time needed for attachment and confluent in supernatant and pellet culture, and primary culture yield

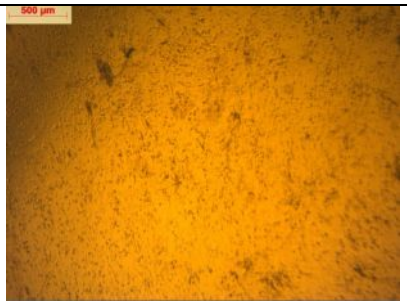
Sample (volume)	Time			Primary culture yield (viable cells)		
	Fibroblastic cell attachment	Confluent		Supernatant	Pellet	Total
		Supernatant	Pellet			
1 (10 mL)	6 days	13 days	18 days	10,800	360,000	370,800
2 (10 mL)	6 days	13 days	15 days	57,600	136,000	193,600
3 (12 mL)	6 days	13 days	20 days*	194,400	134,000	328,400
*extensive washing of contaminating fat and hematopoetic cells caused some attached MSCs to detach						



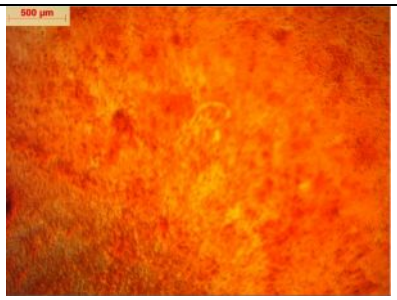
Adipogenic differentiation 400x
Bone marrow derived MSCs



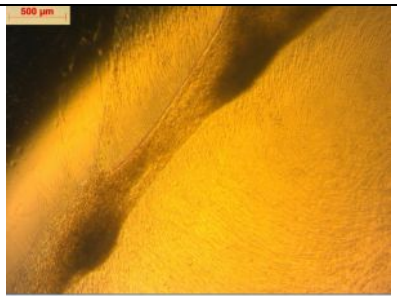
Adipogenic differentiation-oil red- 400x
Bone marrow derived MSCs



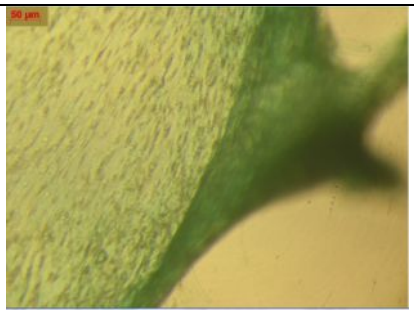
Osteogenic differentiation 40x
Bone marrow derived MSCs



Osteogenic differentiation-alizarin red- 40x
Bone marrow derived MSCs



Chondrogenic differentiation – unstained -40x
Bone marrow derived MSCs



Chondrogenic differentiation – alcian blue-200x
Adipose tissue derived MSCs

Fig. 1. P-I induced cells into adipocytes, osteocytes and chondrocytes

In this study we used xeno-free material for the isolation and harvest of bone marrow derived MSCs. After centrifugation, the supernatant still contained MSCs, and was cultured separately from the pellet. As pellet culture was covered by a thick layer of red blood cells, attachment of MSC can not be seen, and attachment in supernatant culture was used as a sign that MSCs were already attached, and the pellet culture could be washed, which was followed by medium change. Moreover, the supernatant yielded additional amount of cells with variable yield. In sample-3, supernatant culture yielded more than pellet culture.

A study showed that gradient centrifugation separated mononuclear cell primary culture in 10% FBS yielded 2,000,000 MSCs from 50 ml bone marrow aspirate,[19] a little higher compared to our result, when both supernatant and pellet culture results were combined . Most studies on bone marrow derived MSC isolation used fibroblastic colony forming unit (CFU-F) as their outcome measure.[20-23] Therefore, it is difficult to compare to our study, as our study measured viable cell number, while a CFU-F might contain variable number of cells, which depended on the colony size. Colony size may range from small (10-50 cells), medium size (50-250 cells), and large (more than 250 cells).[22]

A study on gradient centrifugation and serum free commercial medium showed that yield of passage-1 was 100,000 MSCs/ml of bone marrow aspirate,[20] much lower than our result for sample-1. Bernardo et al (2007) study that used freezed-thawed PRP as in our study showed that platelet lysate containing medium was superior compared to FBS containing medium for clonogenicity and proliferative capacity of MSCs.[21] Horn et al (2010) showed no significant difference between clonogenicity of 10% PRP lysate and 10% FBS containing medium, but 10% PRP lysate containing medium showed more large clone proportion compared to 10% FBS containing medium. However, there were differences between PRP batches in term of proliferation rate that was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[23] Variation in PRP batches can be minimized by pooling the batches.

Our study showed that there were donor variation in the yield of primary culture of bone marrow derived MSCs, and this result was corroborated by the result of Phinney et al (1999), who found that there were variations between donors, and the highest was twelve fold the lowest yield. Phinney et al study used iliac crest bone marrow samples,[24] while in our study we used two iliac crest and one femoral fracture derived bone marrow samples. Moreover, Phinney et al showed that MSCs from different donors has different osteogenic capacity, and suggested that the MSCs were a mixture of progenitor cells at various stages of differentiation.[24]

Our study showed that the P1-cells from the pellet fraction that were isolated by PRP containing medium were MSCs as they could be induced into adipocyte, chondrocyte and osteocyte lineage. This result was in line with other studies using PRP supplemented media,[21,23] though further researches are needed to elucidate the surface markers, and possibility of subtle differences in differentiation potentials between donors, supernatant versus pellet culture, and PRP batches.

Conclusion

Platelet rich plasma supplemented α MEM can be used to isolate MSCs from bone marrow using simple centrifugation method.

Acknowledgement

This study was funded by the research grant from Directorate of research and public service Universitas Indonesia (Hibah klaster riset 2014), contract number: 1707/H2.R12/HKP.05.00/2014. We are greatly indebted to the residents, doctors, and paramedical staff of Department of Orthopaedic and Traumatology, Cipto Mangunkusumo Hospital, Jakarta, who helped in taking the samples, and the patients, who donated the bone marrow samples.

References

1. Martin-Rendon E, Brunskill S, Doree C, Hyde C, Mathur A, Stanworth S,et al. Stem cell treatment for acute myocardial infarction. Cochrane Database of Systematic Reviews 2008, Issue 4. Art. No.: CD006536.
2. Boncoraglio GB, Bersano A, Candelise L, Reynolds BA, Parati EA. Stem cell transplantation for ischemic stroke. Cochrane Database of Systematic Reviews 2010, Issue 9. Art. No.: CD007231.
3. Ra JC, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, et al. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. Stem Cells Dev. 2011;20:1297-308.
4. Philippe B, Luc S, Valérie PB, Jérôme R, Alessandra BR, Louis C. Culture and use of mesenchymal stromal cells in phase I and II clinical trials. Stem Cells Int. 2010;2010:503593.
5. Hauzeur JP, Gangji V. Phases 1-3 clinical trials using adult stem cells in osteonecrosis and nonunion fractures. Stem Cells Int. 2010;2010:410170.
6. Bakondi B, Shimada IS, Perry A, Munoz JR, Ylostalo J, Howard AB, et al. CD133 identifies a human bone marrow stem/progenitor cell sub-population with a repertoire of secreted factors that protect against stroke. Mol Ther. 2009;17:1938-47.
7. Puglisi MA, Tesori V, Lattanzi W, Piscaglia AC, Gasbarrini GB, D'Ugo DM, et al. Therapeutic implications of mesenchymal stem cells in liver injury. J Biomed Biotechnol. 2011;2011:860578.
8. Lin G, Qiu X, Fandel T, Banie L, Wang G, Lue TF, et al.Tracking intracavernously injected adipose-derived stem cells to bone marrow.Int J Impot Res. 2011;23:268-75.
9. Bieback K, Kinzebach S, Karagianni M. Translating research into clinical scale manufacturing of mesenchymal stromal cells. Stem Cells Int. 2010;2010:193519.
10. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther. 2004;9:747–56.
11. Pawitan JA. Platelet rich plasma in xeno-free stem cell culture: the impact of platelet count and processing method. Curr SC Res Th. 2012;7: 329-35.
12. Sari P, Luviah E, Nugraha Y, Pawitan JA, Lilianty J, Purwoko RY. Various growth factor yields in various platelet rich plasma processing methods. Biotechnology: An Indian Journal. 2014; 9:475-8.
13. Cho HS, Song IH, Park SY, Sung MC, Ahn MW, Song KE. Individual variation in growth factor concentrations in platelet-rich plasma and its influence on human mesenchymal stem cells. Korean J Lab Med. 2011;31:212-8.
14. Huang Q, Wang YD, Wu T, Jiang S, Hu YL, Pei GX. Preliminary separation of the growth factors in platelet-rich plasma: effects on the proliferation of human marrow-derived mesenchymal stem cells. Chin Med J (Engl). 2009;122:83-7.
15. Kurita M, Aiba-Kojima E, Shigeura T, Matsumoto D, Suga H, Inoue K, et al. Differential effects of three preparations of human serum on expansion of various types of human cells. Plast Reconstr Surg. 2008;122:438-48.
16. Kakudo N, Minakata T, Mitsui T, Kushida S, Notodihardjo FZ, Kusumoto K.. Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. Plast Reconstr Surg. 2008;122:1352-60.
17. Phedy, Dilogo IH, Jusuf AA, Kholinne E, Efendi Z. Iliac crest and femoral bone marrow as the source of plastic-adherent cells. Med J Indones. 2011;20:100-4.
18. Pawitan JA, Suryani D, Wulandari D, Damayanti L, Liem IK, Purwoko RY. Prolonged culture in FBS and FBS-substitue containing media: Spontaneous chondrogenic differentiation of adipose tissue derived mesenchymal stem cells. International Journal of PharmTech Research. 2014;6:224-35.
19. Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci U S A. 2002;99:8932-7.

20. Grisendi G1, Annerén C, Cafarelli L, Sternieri R, Veronesi E, Cervo GL, et al. GMP-manufactured density gradient media for optimized mesenchymal stromal/stem cell isolation and expansion. Cytotherapy. 2010;12:466-77.
21. Bernardo ME, Avanzini MA, Perotti C, Cometa AM, Moretta A, Lenta E, et al. Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute. J Cell Physiol. 2007;211:121-30.
22. Horn P, Bork S, Diehlmann A, Walenda T, Eckstein V, Ho AD, et al. Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. Cytotherapy. 2008;10:676-85.
23. Horn P, Bokermann G, Cholewa D, Bork S, Walenda T, Koch C, et al. Impact of individual platelet lysates on isolation and growth of human mesenchymal stromal cells. Cytotherapy. 2010;12:888-98.
24. Phinney DG, Kopen G, Righter W, Webster S, Tremain N, Prockop DJ. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. J Cell Biochem. 1999;75:424-36.

*****