Comparison of fracture site callus with iliac crest bone marrow as the source of plastic-adherent cells

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Abstrak

Latar belakang: *Sumsum tulang merah merupakan sumber utama sel punca mesenkim walaupun penggunaannya menimbulkan morbiditas situs donor. Pengambilan sel punca dari sumsum tulang menyebabkan nyeri dan seringkali sukar dilakukan sehingga membutuhkan sumber alternatif. Karena penyembuhan tulang sekunder terjadi melalui pembentukan kalus hasil proliferasi dan diferensiasi sel punca, kalus mungkin menjadi sumber alternatif pengambilan sel punca mesenkim. Penelitian ini membandingkan jumlah* plastic-adherent cells *dari kalus dan sumsum tulang setelah dua minggu kultur sel.*

Metode: *Enam belas kelinci Selandia Baru dilakukan prosedur frakturisasi diafisis tulang femur. Lalu, seluruh kelinci dirawat. Selanjutnya, dua minggu pasca-frakturisasi, 3 mL aspirasi sumsum tulang krista iliaka dan ekstraksi kalus situs fraktur pada delapan kelinci dilakukan kultur (kelompok I). Delapan kelinci lainnya dilakukan hal yang sama pada empat minggu pasca-frakturisasi (kelompok II). Seluruh kultur diamati setelah satu dan dua minggu. Setelah empat minggu, kultur dipanen. Jumlah sel dihitung dengan hemositometer Neubauer. Kemudian, perbandingan jumlah sel dianalisis menggunakan uji t tidak berpasangan.*

Hasil: *Pada kelompok I terdapat jumlah sel sebanyak 2,6 ± 0,1 x 10^4 untuk kultur aspirat sumsum tulang krista iliaka dan 2,5 ± 0,1 x 10^4 untuk kultur ekstraksi kalus situs fraktur. Tidak terdapat perbedaan bermakna secara statistik antara keduanya (p = 0,34). Sedangkan pada kelompok II didapatkan hasil sebesar 2,7 ± 0,1 x 10^4 sel dan 2,1 ± 0,1 x 10^4 sel secara berurutan dan terdapat perbedaan yang bermakna secara statistik antara keduanya (p < 0,001).*

Kesimpulan: *Situs kalus fraktur dua minggu pasca-frakturisasi memiliki potensi sebagai situs donor untuk isolasi dan ekspansi* plastic-adherent cells. ***(Med J Indones. 2013;22:70-5)***

Abstract

Background: Red marrow has been described as the main source of mesenchymal stem cells although its aspiration and isolation from bone marrow was reported to have significant donor site morbidity. Since secondary bone healing occurs through formation of callus as the result of proliferation and differentiation of mesenchymal stem cells, callus may become alternative source for mesenchymal stem cells. In this study, we compared the number of plastic-adherent cells from fracture site callus and bone marrow of iliac crest after two and four weeks of culture.

Methods: Sixteen New Zealand rabbits were fracturized at the femoral shaft. Then, these rabbits were taken care. After two weeks of fracturization, 3 mL iliac crest bone marrow aspiration and callus extraction of eight rabbits were cultured (group I). The other eight rabbits were treated equally after four weeks of fracturization (group II). Simultaneously, the cultures were observed after one and two weeks. Four weeks later, they were harvested. Cells were counted using Neubauer hemocytometer. The average number of cells between the sources and groups were statistically analyzed using the unpaired t-test.

Results: In group I, there were 2.6 ± 0.1 x 10^4 cells in the culture of iliac crest bone marrow aspirate and 2.5 ± 0.1 x 10^4 cells in culture of callus extract from fracture site (p = 0.34). In group II, there were 2.7 ± 0.1 x 10^4 cells and 2.1 ± 0.1 x 10^4 cells, respectively (p < 0.001).

Conclusion: Fracture site callus at the second week post-fracturization may be potential as source of plastic-adherent cells compared with iliac crest bone marrow. ***(Med J Indones. 2013;22:70-5)***

Keywords: *Bone marrow, fracture site callus, iliac crest, long bone, mesenchimal stem cells, plastic-adherent cells*

Red marrow has been described as the main source of mesenchymal stem cells (MSCs).[1,2] Although red marrow can be easily obtained from iliac crest, the aspiration and isolation from bone marrow of iliac crest have been reported to have significant donor site morbidity.[3] In order to reduce donor site morbidity in non-union fracture, another source of MSCs should be sought.

Non-union long-bone fracture, as diaphysis femur, requires advance surgery intervention which nowadays reached 12.5% of surgeries.[4] The rate of non-union fracture was higher in open tibia fracture. It happened in 12.1% for Gustillo type I injuries to 49.2% in patients with Gustillo type III B.[5]

Many studies reported the success of MSCs in term of bone formation for fracture cases, especially non-union.[6,7] By using Giant Flamish rabbits, Phedy et al[3] found that bone marrow from femoral shaft served as good as bone marrow from iliac crest as source of

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plastic-adherent cells, presumed as MSCs. However, in clinical setting, obtaining marrow from femoral shaft could be challenging in technique.

MSCs are of keen interest because they are potential for treatment of many disorders and injuries such as non-union fracture, bone defect, cartilage injury, pseudoarthtosis, and burn wounds.[8-11] MSCs also have potential role in regenerative medicine.[12]

Secondary bone healing occurs through formation of callus. Since callus is formed from proliferation and differentiation of MSCs, we hypothesized that callus was also potential as source of MSC. In the following study, we evaluated fracture site callus as source of MSC by comparing number of plastic-adherent cells from the fracture site callus and iliac crest after four weeks of culture.

METHODS

Ethical clearance was obtained from the Ethical Committee of Faculty of Medicine Universitas Indonesia prior to the study. We included sixteen male New Zealand rabbits weighted ± 2000 g, with approximately three-month-old.

Fracturization of the femur bone was done manually using bone saw. The femoral diaphysis was exposed using posterolateral approach and then it was fracturized. The procedures were done after the rabbits were anesthetized. After the surgery was done, bandage, and external splint were used at the fracture site. Next, we took care the rabbits by giving standardized diet and drugs in constant temperature and humidity. In that period, we randomly allocated the rabbits into two groups using simple random table. Both iliac crest bone marrow aspirate and callus extract from fracture site were performed after two weeks of fracturization in group I and four weeks in group II.

Iliac crest bone marrow aspiration

Aspiration was performed using needle catheter 14G which was connected to 5 mL-syringes. The catheter was inserted to the iliac crest at 45° of angle to aspirate 3 mL of bone marrow. After the aspirate had been contained in a sterile 10 mL-vacutainer tube, it underwent the culture.

Femur fracture site callus extraction

Callus extraction was performed by open surgery. After the fracture site had been exposed through the previous surgical scar, we identified the callus and extracted it using sterile pincer (Aesculap, US). The callus was then mechanically crushed using sterile mortar. Then, 3 mL callus was taken for culture.

Culture of aspirate and extract

Cells culture was performed at the Institute of Human Virology and Cancer Biology, Jakarta during July and October 2011. The amount of 3 mL bone marrow aspirate and callus extract were diluted 1:1 with phosphate buffered saline (PBS) and centrifuged at 2,500 rpm for 15 minutes at 20°C. Then, the pellet was resuspended into a 75 cm^2 (TC) flasks in low glucose Dulbecco's modified Eagles medium (DMEM; Gibco, Grand Island, New York) containing 1 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, and 10% fetal bovine serum (Gibco, Grand Island, New York); and incubated at 37°C, O_2 flow of 20% and CO_2 of 5%.[9] At the end of the first week, non-adherent cells were discarded and adherent cells were thoroughly washed twice with PBS. Fresh complete medium was added and replaced every three days.

Data collection and analysis

We observed the culture under microscope after one, two, and four weeks. After four weeks, the culture was harvested and the cells from each donor site were counted with Neubauer improved technique. Calculation was done twice by two researchers working independently in blinded manner. Average of cell count from each researcher was used in statistical analysis. Unpaired t-test was performed using SPSS v.16 (Apache Software Foundation, America) to compare cell counts between groups.

RESULTS

At the first week of culture, after discarding the non-adherent cells, some cells were left adhered in the tissue culture flask. These cells were apparent in both sources and both groups. They were round in shape (Figure 1).

At the second week of culture, fibroblast-like cells were observed in culture of bone marrow aspirate both in group I and II (Figure 2). These cells were not observed in culture of callus extract from both group and only round shaped cells were present (Figure 3).

After four weeks of culture, the density of the cells in both sources and groups was increased. However, the cells from callus extract remained round in shape (Figure 4). On cell counts, we obtained $2.6 \pm 0.1 \times 10^4$ cells from culture of iliac crest aspirate and $2.5 \pm 0.1 \times 10^4$ cells from culture of callus extract in group I, while $2.7 \pm 0.1 \times 10^4$ cells and $2.1 \pm 0.1 \times 10^4$ cells in group II, respectively.

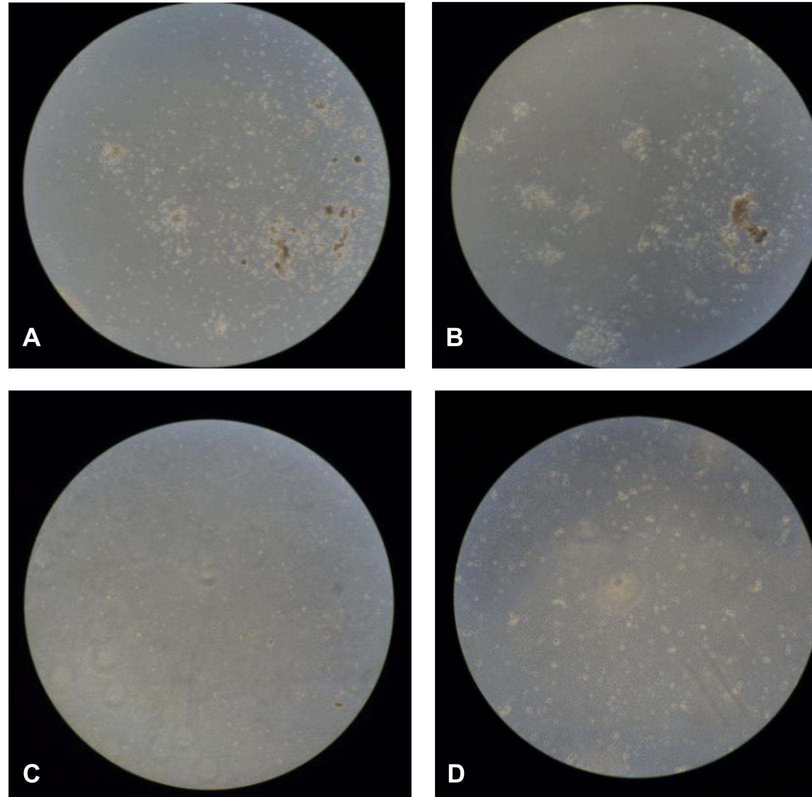

Figure 1. Sample of the first week culture of iliac crest bone marrow aspiration from group I (A) and group II (B). First week culture of fracture site callus extraction from group I (C) and group II (D). Plastic-adherent cells seen in TC-flask

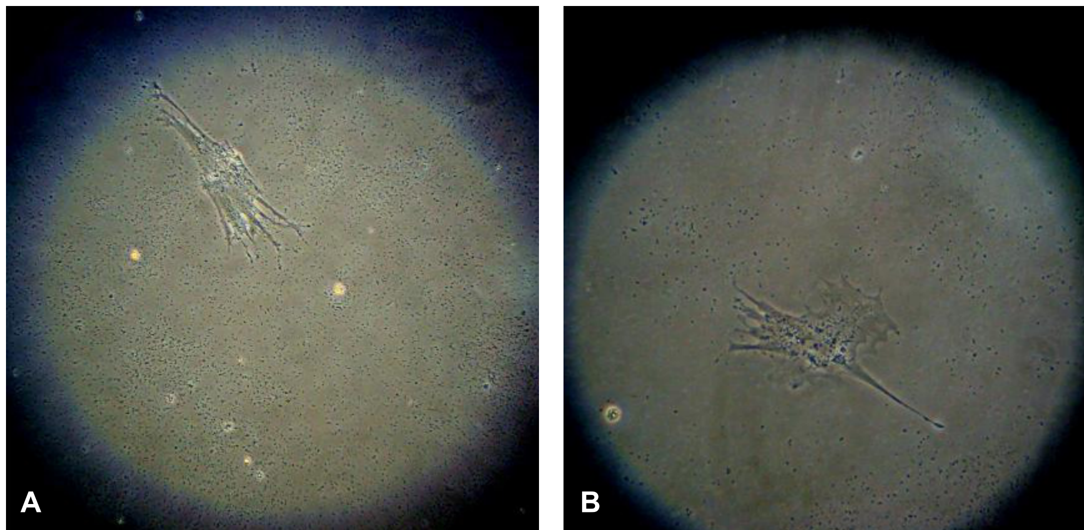

Figure 2. Sample of second week culture of iliac crest bone marrow aspiration from group I (A) and group II (B). Cell growth with fibroblast-like morphology was seen

Statistical analysis between each sources in group I showed no significant difference ($p = 0.34$). The analysis of group II was statistically significant with $p < 0.001$. The significant difference ($p < 0.001$) was also found between group I and II of callus extract.

DISCUSSION

We observed iliac crest bone marrow culture and fracture callus extract culture at the second and fourth week after fracturization as the source of plastic-adherent cells and

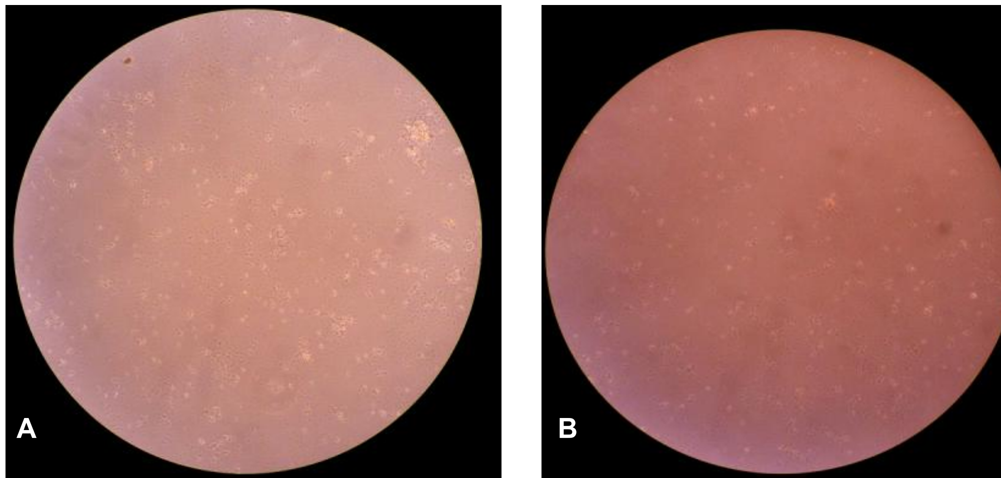

Figure 3. Second week culture of fracture site callus extraction from group I (A) and group II (B). Plastic-adherent cells were seen

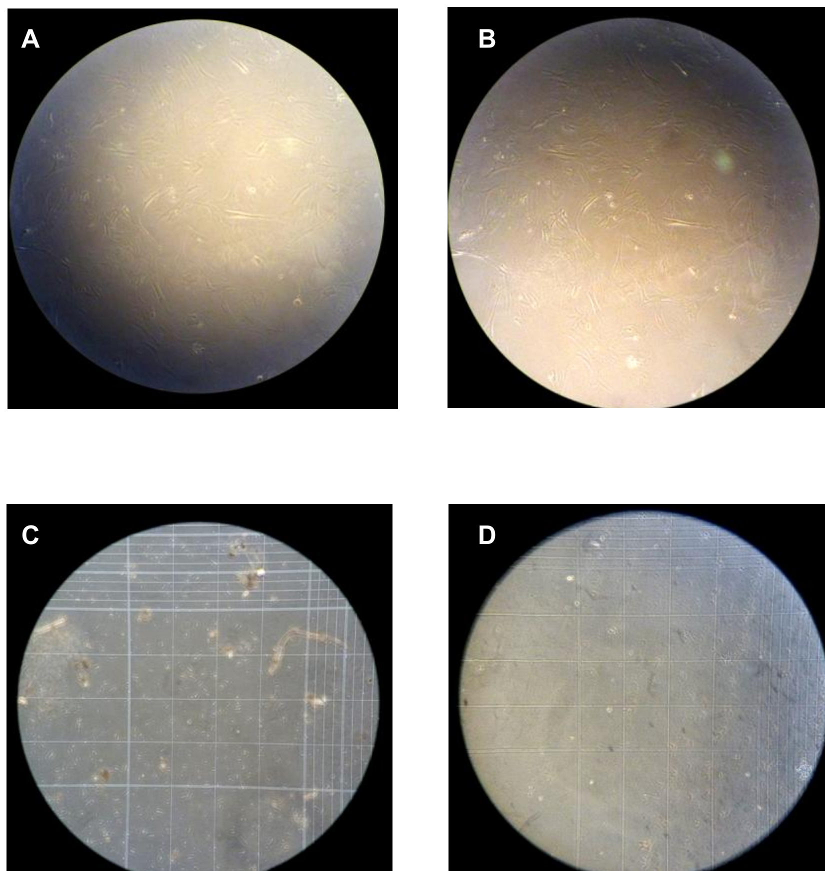

Figure 4. Fourth week culture of iliac crest bone marrow aspiration from group I (A) and group II (B). Fourth week culture of fracture site callus extraction from group I (C) and group II (D). Growth density of fibroblast-like cells in iliac crest aspiration culture and plastic-adherent cells in fracture site callus extraction culture

found similar result between iliac crest bone marrow and fracture callus at group I (2-week post fracturization).

In clinical setting, percutaneous aspiration of bone marrow from iliac crest to obtain MSC is a common procedure. On the other hand, from our literature searching, none has used fracture site callus as the source of MSC.

Since culture of MSC from fracture site callus had never been done before, no standardized method of

callus extraction is available. The method of callus extraction in our study is therefore experimental.

In this study, we performed two different procedures in obtaining the MSC, the bone marrow aspiration from iliac crest and fracture site callus extraction. Iliac crest bone marrow aspiration was performed percutaneously, while obtaining fracture site callus was performed by open surgery. Fennema, et al[13] reported that different strategy of bone marrow aspiration may cause different quantity of MSCs collected.

In addition to the limited literature on using callus as the source of MSC, the optimal period of time for callus to be taken as source of MSC is still unknown. Therefore, we performed extraction from callus at two and four week post fracturization. Moreover, fracture healing process of rabbit as an animal model could be different with human. So, the extraction time to obtain callus from fracture would need further adjustment in order to obtain better result in human.

In this study, the hard callus was clinically found in the four week post-fracturization. The osteoblast and osteocyte differentiation could have occurred during that time.[14] This condition could explain the result which showed cells found in the four week post-fracturization less than two week post-fracturization callus culture that may represent the soft callus. Furthermore, we suggest to use specific marker to confirm the cell characters.

In this study, 3 mL of bone marrow was aspirated. Fennema et al,[13] reported that at least 8 mL volumes of bone marrow aspirate was required to have optimum number of cells obtained. However, the research is valid only for human subject. There is no evidence about recommended volume aspiration for animal models.[15] Our result showed cells fewer than other studies. Phedy, et al[3] showed similar amount of plastic-adherent cells on giant flemmish rabbits. Both, et al[16] showed result of 200 million humans MSC in 12-21 days of culture. Colleoni et al[15] showed more than 60,000 horse cells after one week of culture. These differences bring out the species-dependent feature on the ability of MSC expansion.

Despite similar cell counts between each source in group I, it should be noticed that the cells from each sources were different in morphology. Although the International Society for Cellular Therapy does not regard fibroblast-like morphology as a criterion for definition of MSC, it is generally accepted that MSC morphology is fibroblast-like.[17] It is the limitation of our study that we did not evaluate further on other criteria for definition of MSC such as the specific expression of surface antigen and the multi-potent differentiation capacity into MSC lineage such as osteoblast, adipocyte, and chondroblast on standard *in vitro* differentiation condition.

In conclusion, this preliminary study showed the possibility of fracture site callus as potential source for plastic-adherent cells. Although, these cells warrant further analysis to be defined as MSCs.

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