

## Iliac crest and femoral bone marrow as the source of plastic-adherent cells

Phedy,<sup>1</sup> Ismail H. Dilogo,<sup>2</sup> Ahmad A. Jusuf,<sup>3</sup> Erica Kholinne,<sup>1</sup> Zuhri Efendi<sup>1</sup>

<sup>1</sup> Resident at Departemen of Orthopaedic dan Traumatology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

<sup>2</sup> Consultant at Departemen of Orthopaedic dan Traumatology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

<sup>3</sup> Consultant at Department of Histology, Faculty of Medicine, University of Indonesia

### Abstrak

**Latar belakang:** Banyak penelitian yang menggunakan krista iliaka sebagai donor sel punca mesenkimal. Pada kasus fraktur tulang panjang, pengambilan sumsum tulang dari situs fraktur memberikan banyak keuntungan. Akan tetapi, sumsum tulang panjang yang tergolong sebagai sumsum kuning mengandung banyak sel lemak sehingga dipercaya mengandung jumlah sel punca yang lebih sedikit daripada sumsum merah. Karena itu, penelitian ini bertujuan untuk membandingkan potensi sumsum merah dan sumsum kuning sebagai donor sel punca mesenkimal.

**Metode:** Sumsum tulang dari 8 ekor kelinci Flemish giant jantan diaspirasi dari krista iliaka dan batang femur. Sel mononuklear diisolasi dari aspirat dan diekspansi dalam medium DMEM rendah glukosa. Setelah 8 minggu, sel dipanen dan dihitung dengan hemositometer Neubauer. Perbandingan jumlah sel antara kedua situs dianalisis dengan menggunakan uji t-test.

**Hasil:** Setelah 8 minggu, sejumlah rata-rata  $2,93 \pm 0,91 \times 10^4$  dan  $3,7 \pm 2,50 \times 10^4$  sel diperoleh dari kelompok krista iliaka dan batang femur. Tidak ada perbedaan statistik yang bermakna antara kedua kelompok. ( $p = 0,45$ ).

**Kesimpulan:** Sel yang melekat pada plastik dapat diisolasi dan diekspansi dari krista ilaka maupun batang femur. (*Med J Indones 2011; 20:100-4*)

### Abstract

**Background:** Many studies have used iliac crest as the source of mesenchymal stem cells. In cases of long bone shaft fracture, obtaining marrow from the fracture site offers more advantages. Nevertheless, due to the high number of fat cells in long bones, the yellow marrow of long bones is believed to contain lower number of mesenchymal stem cells than red marrow. Therefore the aim of this study is to compare the potency between red and yellow marrow as the donor site for the isolation of mesenchymal stem cell.

**Methods:** Bone marrow of eight giant Flemish rabbits was aspirated from the iliac crest and femoral shaft. Mononuclear cells were isolated from both aspirates and expanded in low glucose DMEM. After eight weeks, the cells were harvested and counted using improved Neubauer hemocytometer. Comparison of the cell number between the two donor sites was then performed by t-test.

**Results:** After 8 weeks, an average number of  $2.93 \pm 0.91 \times 10^4$  and  $3.7 \pm 2.50 \times 10^4$  cells were obtained from the iliac and femoral group respectively. No statistically significant difference was found between those two groups ( $p = 0.45$ ).

**Conclusion:** Plastic-adherent cells can be isolated and expanded from both iliac crest and femoral shaft. (*Med J Indones 2011; 20:100-4*)

**Key words:** plastic-adherent cells, red marrow, yellow marrow

Many studies reported mesenchymal stem cell (MSC) to play an important role in fracture healing.<sup>1,2</sup> Lee et al. reported improved bone healing in non-union fracture in a dog,<sup>1</sup> while Kon et al. reported bone healing acceleration in critical-size defects of sheep long bones.<sup>2</sup> Most studies didn't report the source of the stem cells, but some of them used red marrow.<sup>1,3</sup> Although red marrow can be obtained easily from the iliac crest, in the case of long bone shaft fracture however, obtaining marrow from the fracture site offers more advantages. It requires less additional procedure, faster, and eliminates donor site morbidity.

Unfortunately, marrow of the long bone is considered as yellow marrow. Yellow marrow contains more lipid than stromal cells and therefore is believed to have lower potency compared to red marrow if it is used as the source of stem cells. Nevertheless, to our knowledge, no study has addressed the issue. We therefore compared

the potency of yellow to red marrow as the source of mesenchymal stem cells.

### METHODS

Ethical clearance was obtained from the Ethical Committee of Faculty of Medicine, University of Indonesia prior to the study. Eight giant Flemish rabbits weighted  $\pm 2900$ g (Table 1) were included in the study.

#### Bone marrow aspiration procedure

After anesthetized, the femoral shaft was surgically exposed and the cortex was drilled using a 1.5 drill bit until it was thin enough for a 14-gauge syringe to be inserted to aspirate one ml of its marrow. Another one ml of marrow was aspirated using 14-gauge syringe percutaneously from the iliac crest.

### Culture of aspirated cells

Cell culture was performed at the Institute of Human Virology and Cancer Biology, Jakarta during February and March 2010.

Each aspirate was diluted 1:1 with phosphate buffered saline (PBS) (Gibco, Grand Island, New York) and centrifuged at 2500 g for 15 minutes at 20°C. The pellet was then resuspended into a 25 mL TC-flask in 8 ml low glucose Dulbecco's modified Eagles medium (Gibco, Grand Island, New York) containing 1g/L D-glucose, L-glutamine, 110mg/L sodium pyruvate, and 10% fetal bovine serum (Gibco, Grand Island, New York); and incubated at 37°C, O<sub>2</sub> flow of 20% and CO<sub>2</sub> of 5%. The cultures were examined after one week, and every three days. At the end of the first week, non-adherent cells were discarded and the adherent cells were thoroughly washed twice with PBS. Fresh complete medium was added and replaced every 3 days.

### Data collection and analysis

After 8 weeks, the culture was harvested and cells from each donor site were counted blindly using improved Neubauer hemocytometer. The data was noted, tabulated and mean±SD for each group was calculated. A student t-test was performed using STATA v.10 (StataCorp, Texas, Amerika) to compare the mean between those two groups.

### RESULTS

At the time of culture, mononuclear cells were covered by red blood cells (Figure 1). After discarding nonadherent cells, only cells adhered to plastic remained. They exhibited fibroblast-like morphology. After 4 week culture, the number of cells increased in both groups (Figure 2). The density of the cells increased rapidly until the eighth week (Figure 3). At the eighth week, the culture yielded  $2.93 \pm 0.91 \times 10^4$  and  $3.66 \pm 2.50 \times 10^4$  cells for the iliac crest and femoral shaft respectively. Cell counts for each donor site were shown in table 1. Statistical analysis showed no difference between those donor sites ( $p = 0.45$ ).

Table 1. Weight of the donor and cell counts after 8 weeks of culture (in 10<sup>4</sup> cells)

Weight (g)	Cell count	
	Iliac crest	Femoral shaft
2100	4	5
2250	1.25	1.25
3750	2.5	1.25
3500	3.5	6.75
2800	3.75	7.5
3350	3.4	1.5
3000	2.5	3.5
2450	2.5	2.5
Mean	2.93	3.66

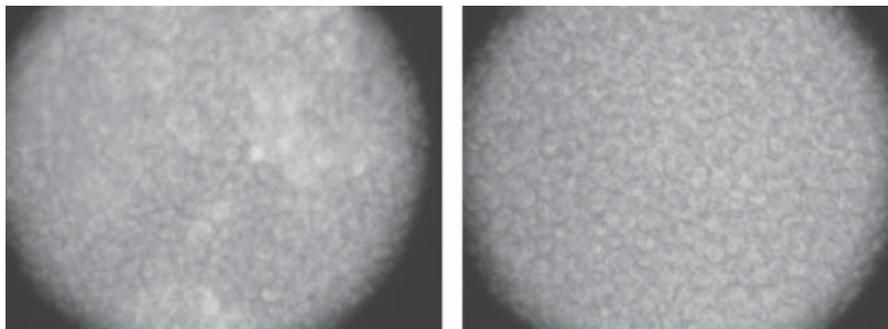


Figure 1. Cells at the time of culture: left= iliac crest, right= femoral shaft. At the time of culture, the mononuclear cells were covered by red blood cells. All magnifications  $\times 40$ .

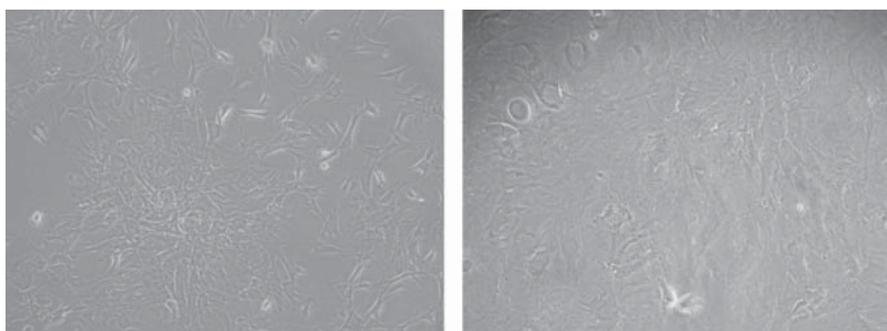


Figure 2. Cells 4 weeks after culture: left= iliac crest, right= femoral shaft. Morphological changes toward spindle-shape were observed in both groups. All magnifications  $\times 40$ .

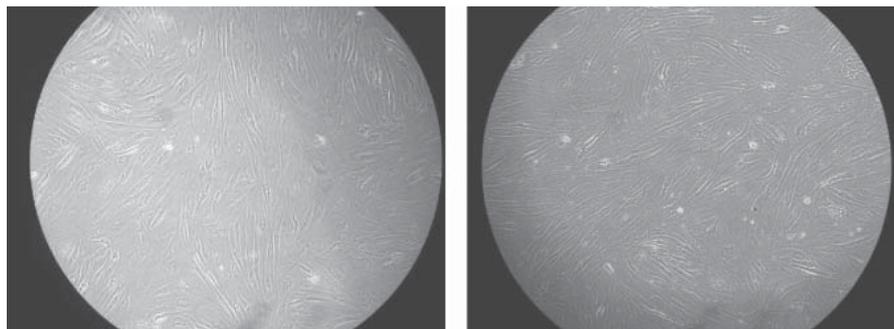


Figure 3. Cells 8 weeks after culture; left=iliac crest, right=femoral shaft. At the 8th week, spindled-shaped cells adhered to plastic were observed in both groups. Both had high density. All magnifications are 40 times.

## DISCUSSIONS

In our study, the two procedures by which we performed marrow aspiration slightly differed, i.e. aspiration from the iliac crest was performed percutaneously, while for femoral shaft was done by surgery. Fennema et al. reported that different strategy of bone marrow aspiration would lead to different quantity of mesenchymal stem cells.<sup>4</sup> In real clinical settings, it is possible to obtain a percutaneous aspiration of marrow from the iliac crest. On the other hand, percutaneous marrow aspiration from intact femoral shaft is almost impossible due to the thick cortices of the bone, except in the cases of fracture where the marrow of the bone is usually exposed.

We observed that closed aspiration of bone marrow from the iliac crest resulted in more bleeding than open aspiration of bone marrow from the femoral shaft. Since concentration of the mesenchymal stem cells in peripheral blood is much lower than that in the bone marrow, the yield of MSC will definitely lower if more blood is aspirated.<sup>4</sup>

Fennema et al. also reported that at least 8ml collection volumes of bone marrow aspirates were required to reduce the risk of obtaining aspirates with lower cell numbers.<sup>4</sup> However, this result is only valid for human subject. In animal models, no study has reported the recommended volume of aspiration to yield adequate MSC. In our study, only 1mL of bone marrow was aspirated.

In this study, we were able to isolate the plastic-adherent cells from both iliac crest and femoral marrow. We supposed that these cells were mesenchymal stem cells, since mesenchymal stem cells adhere to plastic and exhibit fibroblast-like morphology, similar to that observed in our study.<sup>5,6</sup> Iwakura et al. reported that fibroblastic adherent cells were consistently positive for mesenchymal stem cell related markers and differentiation capacity.<sup>7</sup> However, to confirm them as mesenchymal stem cells, surface antigen expression and multi-lineage differentiation capacity should be tested.

Isolation of MSC from the femoral marrow has been investigated before by Leonardi et al. and Caipetti et al.<sup>8,9</sup> Leonardi et al. reported that femoral marrow was highly effective in proliferating and differentiating along the osteogenic lineage.<sup>8</sup> Caipetti et al. found that MSC isolated from the femur of adult patients consistently maintain an osteogenic potential.<sup>9</sup>

In this study, the expansion rate of the cultured cells was lower than that reported by other studies.<sup>10,11</sup> Both et al. reached 200 million cells in 12-21 days for human MSC.<sup>10</sup> In equines, more than 60 000 cells could be achieved after 1 week of culture.<sup>11</sup> The differences might suggest that MSC expansion is species-dependent.

Many factors have been reported to influence MSC expansion rate. Appropriate cells source, culture medium (including fetal calf serum quality, glucose concentration, stable glutamine), hypoxic condition, seeding densities, TC flask size, plastic surface quality, and addition of growth factor may influence the expansion rate.<sup>10-16</sup>

We used low-glucose DMEM (DMEM-LG) as the basal medium of culture in this study. This medium is reported to be able to support MSC growth and maintain population doubling time.<sup>17</sup> Cell confluence could be achieved earlier when cultured in Mesencult®, however, it failed to support human MSCs growth beyond five passages. After 10 passages, the morphology of the MSC cultured in DMEM-LG also significantly changed to a leaf-like, flat and granulated phenotype.<sup>17</sup> The characteristics surface markers expression also decreased.<sup>17</sup> DMEM-LG is also reported to be inferior to  $\alpha$ -MEM in cell number yield.<sup>18</sup> Pal et al. suggested that knockout DMEM and DMEM-F12 supplemented with 10% FBS were the optimum and suitable culture medium for MSC isolation and expansion.<sup>17</sup> Another factor that might take responsibility for the lower expansion rate in our study was the size of the tissue culture flask used to isolate the MSC. Chen et al. compared the usage of 25ml and 75ml flask and found that the best condition for the MSC isolation was achieved using 25ml flask.<sup>16</sup>

In our study, bone marrow of the femoral shaft showed insignificant different potency compared to bone marrow of iliac crest. This finding is contradictory to the common belief that yellow marrow is a poor source of mesenchymal stem cells.<sup>18</sup> If yellow marrow does have the same potency as red marrow as the source of mesenchymal stem cells, obtaining cells from the fracture site offers many advantages. Surgery can be focused on the fracture site and morbidity at the donor site, specifically at the iliac crest, will be eliminated.

Although our findings might reflect that yellow marrow shared the same potency with red marrow as mesenchymal stem cell source, it should be noticed that some bias might influence our study. The procedure by which we obtain the marrow was slightly different. Marrow of the iliac crest was aspirated percutaneously in a blinded fashion, while marrow of the femoral shaft was aspirated openly. Blind aspiration might result in more bleeding, thus less cells were aspirated. This might also be the probable explanation to the finding that more plastic-adherent cells were achieved from femoral shaft than from iliac crest.

Plastic-adherent cell isolation in our study was performed using gravity separation technique without the use of a density gradient. This technique was reported to result in four fold higher mononuclear cell recovery and is less time consuming.<sup>19</sup> However, we couldn't determine the number of mononuclear cells isolated because they were covered by the red blood cells. We therefore couldn't confirm whether the larger number of plastic-adherent cells in marrow isolated and expanded from femoral shaft was due to the larger number of mononuclear cells aspirated at the beginning of the study.

Other limitation to our study is that cell count was performed manually using improved Neubauer hemocytometer. Although the count was performed blindly, it would be more objective if they were counted using computerized equipment. We also did not characterize the cells either by surface antigen expression nor multi-lineage differentiation capacity.

In conclusion, marrow of the femoral shaft contains not less plastic-adherent cells than marrow of iliac crest ( $3.7 \pm 2.50 \times 10^4$  vs.  $2.93 \pm 0.91 \times 10^4$ ). Since it offers many advantages, it may serve as a potential donor site for the isolation and expansion of plastic-adherent cells. However, another well prepared study involving human subject and considering age factor should be performed to confirm our finding.

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