

Osteogenic potency of human bone marrow mesenchymal stem cells from femoral atrophic non-union fracture site

Femoral atrofik birleşmemiş kırık yerinden alınan insan kemik iliği mezenkimal kök hücrelerinin osteogenic potansiyeli

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ABSTRACT

Objective: Mesenchymal stem cells (MSCs) exist in the site of atrophic non-union fracture. The aim of this study was to evaluate the osteogenic potency of MSCs in order to have a better understanding of the unclear pathophysiology of atrophic non-union fracture

Methods: This is an in vitro experimental study. Sample was obtained from the non-union site of a patient with a 6-years-history of atrophic non-union fracture of right femur. The MSCs was isolated from the fracture site and was cultured in the growth medium. Confirmation of the MSCs was performed and then osteogenic differentiation was performed in mono-layered MSC grown in both home-made and commercial osteogenic media. To evaluate the osteogenic differentiation, we performed Alizarin red staining and colorimetric assay for alkaline phosphatase (ALP).

Results: From Alizarin red staining, most cells in the osteoblast medium were stained red by the staining. The result of colorimetric assessment of ALP shows that peak concentration was reached after 4 minutes in osteogenic group and control group.

Conclusion: The presence of ALP activity and positive Alizarin red staining in our study showed that MSCs stem cells obtained from site of atrophic non-union is capable to be differentiated into osteogenic cells. *J Clin Exp Invest* 2014; 5 (2): 159-163

Key words: Osteogenic potency, Mesenchymal stem cell, non-union fracture

INTRODUCTION

Non-union fracture is one of the most difficult complications in orthopaedic. These cases often require readmission and surgery, which exaggerate the cost greatly, with an estimate of £13 844.68 for a single

ÖZET

Amaç: Bu çalışmanın amacı atrofik birleşmemiş kırık patofizyolojisini daha iyi anlamak için bu bölgelerdeki mezenkimal kök hücrelerinin (MKH) osteojenik potansiyelini araştırmaktır.

Yöntemler: Çalışmamız in vitro deneysel bir çalışmadır. Altı yıllık sağ femurda atrofik birleşmemiş kırık öyküsü bulunan bir hastanın kırık yerinden örnek alındı. Mezenkimal kök hücreler kırık bölgesinden izole edildi ve besiyerine kültüre ekildi. Mezenkimal kök hücrelerinin doğrulanma ve daha sonra osteojenik farklılaşması, tek tabakalı MKH ve ticari osteojeik ortamda yapıldı. Osteojenik farklılaşmayı değerlendirmek için Alizarin kırmızı boya ve alkalen fosfataz için kolorimetrik test yapıldı.

Bulgular: Alizarin boyası ile osteoblastik ortamdaki hücrelerin çoğu kırmızıya boyandı. Alkalen fosfatazın kolorimetrik değerlendirilmesi osteojenik ve kontrol grubunda dört dakika sonra pik konsantrasyona ulaştı.

Sonuçlar: Çalışmamızda alkalen fosfataz aktivitesi varlığı ve pozitif Alizarin kırmızı boyanması atrofik birleşmemiş bölgeden alınan MKH'ların osteojenik hücrelere farklılaşma potansiyeli olduğunu gösterdi.

Anahtar kelimeler: Osteojenik potansiyel, mezenkimal kök hücre, birleşmemiş kırık

case of non-union [1,2]. Besides the direct cost, the burden from the decreased productivity and quality of life also occurs, of which it costs \$18 712 USD for one case [3].

Effective treatment can only be done if surgeon understands the underlying pathophysiology

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of non-union [4]. Abundant callus can be found in hypertrophic non-union, indicating ubiquitous blood, oxygen, and nutrient supply. It is considered to be a result of insufficient stability and treatment is directed toward stabilization of the fracture. In atrophic non-union, however, the pathophysiology remains unclear.

Dickson et al suggested poor vascularization as a cause of atrophic non-union [5]. Arterial occlusion in the ipsilateral extremity was associated with a higher rate of delayed union or non-union in open fractures of the tibia. However, Brownlow, Reed and Simpson found that in the animal model, the atrophic non-unions were well vascularized [6].

Centeno et al have reported success in treatment of non-union fractures by percutaneous injection of autologous, culture-expanded, bone marrow-derived mesenchymal stem cells (MSCs). It seemed that lack of MSCs was the cause of the atrophic non-union fracture [7].

In our previous study, we found that MSCs exist in the site of atrophic non-union fracture [8]. However, whether they are capable to differentiate into osteoblast was not evaluated. In the present study, we evaluate the osteogenic potency of MSCs from site of atrophic non-union fracture. Understanding the osteogenic potency of MSCs from site of atrophic non-union will help in better understanding of the unclear pathophysiology of atrophic non-union fracture.

METHODS

This was an *in vitro* experimental study. The study was conducted in Cipto Mangunkusumo Hospital and Regenerative and Cellular Therapy (ReGeniC) laboratory in Jakarta, Indonesia during the periods of July-December 2012. Sample was obtained from the non-union site of a 23-year-old patient with a 6-years-history of atrophic non-union fracture of right femur. During open reduction and internal fixation for atrophic non-union fracture, and before recanalization of the fracture, a 10 ml syringe prefilled with 2 ml heparin 1000 IU/ml was introduced until it reached the medullary canal. The plunger was then pulled to aspirate the marrow. The procedure was repeated until 10 ml of marrow was obtained. The surgery was then continued following the routine procedure.

Equivalent amount of phosphate buffer saline (PBS) was added to marrow aspirate. The solution was centrifuged at 3000 rpm, 20°C for 30 minutes. Pellet was re-suspended into a 75 cm² TC flasks

with growth medium that contained low glucose Dulbecco's modified eagles medium (Gibco, Grand Island, New York) containing 1000 mg/L D-glucose, L-glutamine, 100 mg/L sodium pyruvate, and 10% fetal bovine serum. The suspension was incubated at 37°C, 20% oxygen and 5% CO₂ flow. After 7 days, the culture was washed several times with PBS and the medium was replaced with fresh medium every 3 day until confluence.

Confirmation of mesenchymal stem cells was performed by flowcytometry. MSCs were washed with PBS/2% FBS and incubated for 30 minutes in PE-conjugated mouse monoclonal anti-human CD73 (BD Biosciences, San Jose, CA), PE-conjugated mouse monoclonal anti-CD105 (Abcam, Cambridge, UK), FITC-conjugated mouse monoclonal anti-CD45 (BD Biosciences, San Jose, CA), FITC-conjugated mouse monoclonal anti-human CD34 (BD Biosciences, San Jose, CA), PE-conjugated mouse monoclonal anti-CD19 (Abcam, Cambridge, UK), or PE-conjugated mouse monoclonal anti-HLA-DR+DP+DQ (Abcam, Cambridge, UK), or FITC-conjugated mouse monoclonal anti-CD14 (Abcam, Cambridge, UK) antibody. The expression of corresponding markers were detected with flow cytometry (FACSCalibur, Franklin Lakes, NJ) and analyzed with CellQuestPro Software.

Once the MSCs culture reach confluence, osteogenic differentiation was performed in monolayered MSC grown in both home-made and commercially available osteogenic media (MesenCult®) and incubated 37°C, 20% oxygen and 5% CO₂ flow. Home-made medium contained DMEM, 10% FBS, 1% antibiotics, 0.1 µM dexamethasone, 50 µM ascorbate-2-phosphate, 10mM β-glycerophosphate.

After 7 day, cells culture was washed several times with PBS and the medium was replaced with fresh medium every 3 day until confluence. Confluence in commercially available medium was reached in 5 weeks while all cells in the home-made medium failed to survive.

To evaluate osteogenic differentiation, we performed Alizarin red staining and colorimetric assay for alkaline phosphatase (ALP). Undifferentiated mesenchymal stem cells were used as negative control. Colorimetric assay was performed using Alkaline Phosphatase Colorimetric Assay Kit (Abcam, Cambridge, UK) containing p-nitrophenyl phosphate (pNPP) as a phosphatase substrate. Amount of 25 micro liter of cell lysate was prepared using three cycles of freeze-thaw in de-ionized distilled water. The cells lysate was added to a well plate

with assay buffer and pNPP. The samples were shielded from direct light at room temperature for 1 h. Afterward, L Stop Solution (3N NaOH) was added to the wells and the plate was read at 405 nm in a micro-plate reader.

RESULTS

After 3 weeks, the MSCs were successfully isolated and expanded as observed by BMSCs attachment

with fibroblast-like morphology appearance in a light microscope with 100x magnification. They were adherent to the TC flask and was not washed in the medium replacement. The confluence of cells was 80%-90% with total number of the cells 3.6×10^6 and 2.2×10^6 . (Figure 1) The MSCs characteristic was confirmed with $\geq 95\%$ expression of CD105 and CD73 as well as its low expression ($\leq 2\%$) of CD14, CD34, CD45, CD19, and HLA-DR.

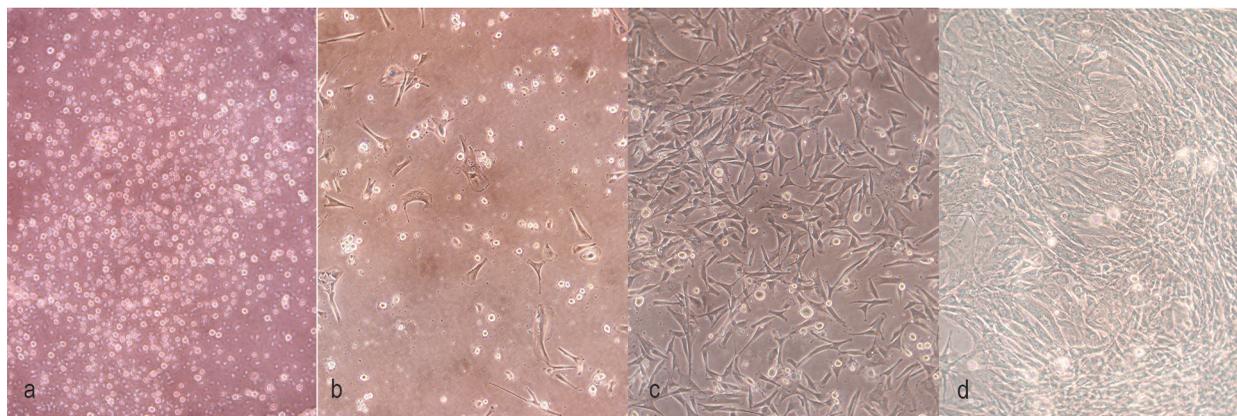


Figure 1. Microscopic evaluation of mesenchymal stem cells (x100).

(a) The first week isolation of BMSCs, the mononuclear cells is adhered to the Tissue Culture Flask undergo periodic medium replacement

(b) On day 5, MSCs were detected with fibroblast-like cells morphology

(c) The end of second week, most of the them are fibroblast like cells adhered to Tissue Culture Flask, minimal mononuclear cells were observed

(d) The end of third week, 90% of cell confluence, indicating the differentiation process to proceed

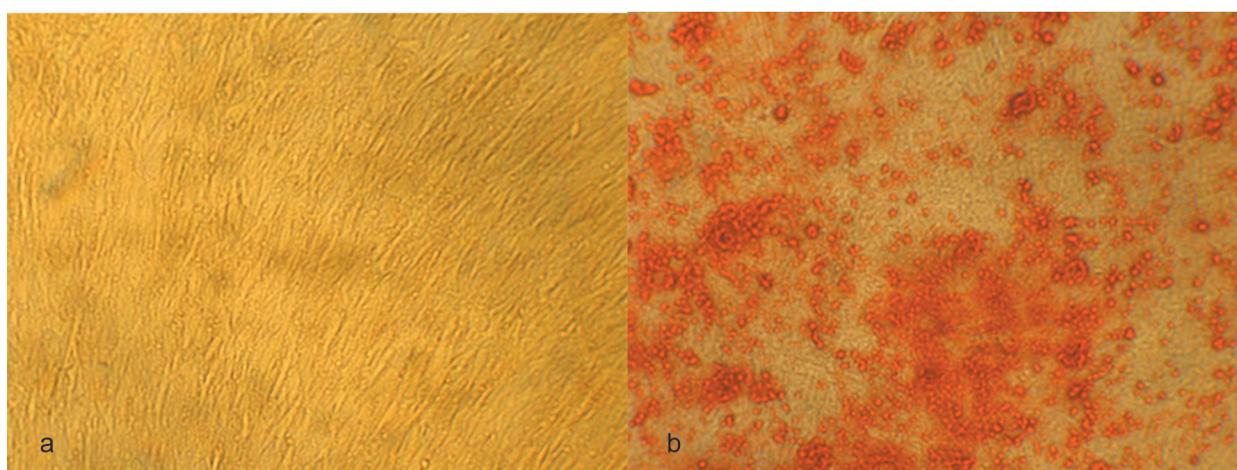


Figure 2. Shows the result of Alizarin red staining of cells in osteogenic medium in comparison to control after 5 weeks of cells culture. Most cells in the osteoblast medium were stained red by the staining

Microscopic evaluation of osteogenic differentiation (alizarin red staining, x100)

(a) control MSCs in growth medium; (b) MSCs in osteogenic medium stained with Alizarin Red

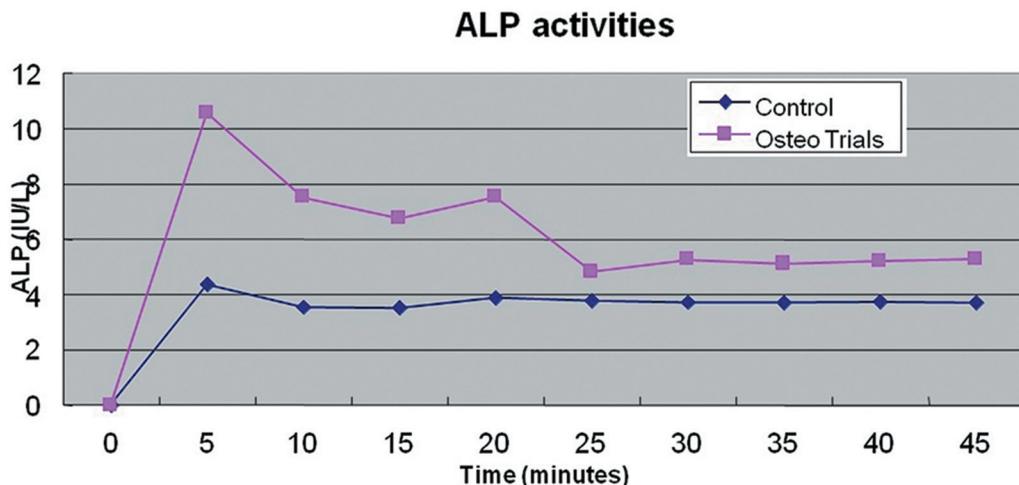


Figure 3. Colorimetric assessment of alkaline phosphatase. Shows the result of colorimetric assessment of ALP. Peak concentration was reached after 4 minutes in osteogenic group and control respectively.

DISCUSSION

In our previous study, we revealed the existence of mesenchymal stem cells in atrophic non-union fracture site [8]. However, in our, we did not evaluate the osteogenic potency of those cells in the study. In this study, we evaluated the osteogenic potency of mesenchymal stem cells in the site of atrophic non-union fractures.

Characterization of cells obtained from the site of atrophic non-union fracture confirmed the presence of mesenchymal stem cells. The cells that we obtained were able to adhere to plastic in standard culture condition, positive expression on CD 105, CD 73, CD 90 for at least 95%, negative expression of CD 45, CD 34, CD 14 or CD 11b, CD 79a, and HLA-DR [9].

Despite similar constituents, we found failure of our home-made medium to induce differentiation of MSCs into osteogenic cells. [10] All cells died before 5 weeks and none was left for further evaluation. The failure might due to the imprecise composition of the medium that influence the milieu. Further researches must be done to evaluate the effect of this homemade osteogenic medium upon its capability in inducing osteogenic differentiation of MSCs. Addition of various chemical substances had been reported to increase the success of osteogenic differentiation [11-13].

Osteogenic differentiation of MSCs can be characterized by various technique, depending on the development stage of the cells; cells proliferation, matrix maturation, and matrix mineralization [14-15].

During cells proliferation, several extracellular matrix proteins can be detected, such as procol-

lagen I, TGF-B and fibronectin. Matrix maturation phase is characterized by maximal expression of alkaline phosphatase. During matrix mineralization, proteins such as osteocalcin, bone sialoprotein, and osteopontin are expressed, Calcium deposition during this phase can also be evaluated using adequate staining methods such as Alizarin red.

Marom et al reported ALP was expressed during early stage of differentiation and was involved with matrix mineralization.[16] Alizarin red is a staining method to determine the presence of calcific deposition by cells of an osteogenic lineage during mineralization phase. It is an early stage marker of matrix mineralization [17-18]. The presence of ALP activity and positive Alizarin red staining in our study showed that MSCs stem cells obtained from site of atrophic non-union is capable to be differentiated into osteogenic cells.

Although our study is limited only to one sample, it is a preliminary result for further evaluation of osteogenic potency of mesenchymal stem cells obtained from site of atrophic non-union. Confirmation of this finding will lead to better understanding of pathophysiology of atrophic non-union and improvement in management strategy.

In conclusion, our preliminary study found the possible osteogenic potency of mesenchymal stem cells obtained from non-union site.

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