

Basic Medical Research

Role of allogenic mesenchymal stem cells in the reconstruction of bone defect in rabbits

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Abstrak

Latar belakang: Meskipun ada banyak penemuan baru untuk rekonstruksi tulang pada dekade terakhir, defek tulang luas tetap menjadi permasalahan di bidang ortopedi. Sel punca mesenkimal (SPM) muncul sebagai salah satu solusi yang berpotensi menangani masalah defek ini. Penelitian ini bertujuan mempelajari efektivitas transplantasi SPM dalam kombinasi dengan hidroksiapatit kalsium sulfat.

Metode: Dua puluh delapan kelinci secara acak dibagi ke dalam empat kelompok. Setiap kelompok mendapat perlakuan yang berbeda: otoplastik, hidroksiapatit kalsium sulfat (HA-CaSO₄), HA-CaSO₄ dikombinasikan dengan aspirat sumsum tulang, atau HA-CaSO₄ dikombinasikan dengan 2x10⁶ SPM. Defek tulang panjang sepanjang 1 cm dibuat, dan langsung difiksasi dengan mini plate-screw dan dua cerclage wires. Tindakan ini diikuti dengan transplantasi graft. Ketebalan kalus diukur dari rontgen yang dikerjakan pada minggu ke-4, -8, dan -12 setelah transplantasi oleh kedua peneliti secara independen. Pada akhir penelitian, pewarnaan histologi serta indeks osteosit diperoleh dengan mengorbankan kelinci. Data tersebut dianalisis dengan uji one-way ANOVA.

Hasil: Ketebalan kalus berbeda secara bermakna pada minggu ke-4 ($p = 0,018$). Walaupun tidak berbeda secara bermakna, kalus pada kelompok SPM di minggu ke-8 tampak lebih tebal daripada kelompok kontrol ($p = 0,546$). Kelompok SPM cenderung menunjukkan indeks osteosit yang lebih tinggi pada minggu keempat dan seterusnya.

Kesimpulan: Transplantasi SPM pada defek tulang menunjukkan formasi kalus yang lebih cepat, dan kalus yang lebih tebal.

Abstract

Background: Despite the advancement of bone reconstruction in the past decade, large bone defect remains a challenge for orthopedic surgery. As mesenchymal stem cells (MSCs) emerges as one of the possible treatment of these defects, we evaluate the effect of its transplantation, particularly in combination with hydroxyapatite-calcium sulphate pellets.

Methods: Twenty eight rabbits were randomly assigned into four different treatment groups. Each group received a different type of grafts (Autograft, hydroxyapatite-calcium sulphate [HA-CaSO₄], HA-CaSO₄ combined with marrow aspirate, or HA-CaSO₄ combined with 2x10⁶ MSCs). One centimeter long bone defects were created then immediately fixated with mini plate-screw and two cerclage wires. It was followed by the graft transplantation. Callus thickness was measured from the x-rays taken at 4, 8, 12 week after transplantation by two authors working independently. At the end of the study, histological staining along with osteocyte index were obtained by sacrificing the rabbits. These data were analyzed by one-way ANOVA test.

Results: At the fourth week, callus thickness showed significant difference ($p = 0.018$). Although statistically insignificant, callus in MSCs group at the eighth week seemed to be thicker than any other groups of intervention ($p = 0.546$). The MSCs group also tend to have a higher osteocyte index at the follow-up weeks.

Conclusion: MSC transplantation on bone defect results in faster callus formation and tends to generate a thicker callus.

Keywords: bone defect, callus thickness, hydroxyapatite-calcium sulphate, mesenchymal stem cells, osteocyte index

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Bone defect remains a challenge for orthopaedic surgeons. It is often complicates fracture and necessitates additional reconstruction procedure.¹ In our center, bone defect-related reconstruction procedures are required in 27% of all fracture cases.

Reconstruction of bone defect can be performed either by using autograft, allograft, or synthetic bone substitute.^{2,3} Autograft has a role as a scaffold and also induces bone healing. However, its availability for treating a large bone defect is very limited. Bone consolidation also occurs slower and causes significant donor site morbidity in some patients.^{1,3} Although allograft is readily available, it carries a higher risk of infection transmission rate, has a higher re-fracture rate, and is difficult to be remodeled.¹ Synthetic bone substitute which created from undegradable materials impairs bone healing and tissue function.² Conversely, the strength of degradable materials is still questionable and influenced by many factors.²

The advancement of tissue engineering has enabled us to isolate and expand mesenchymal stem cells (MSCs), cells that are capable to differentiate into osteoblast cells which is required in bone formation.^{4,5} Therefore it presents the possibility of MSCs to be applied in reconstruction of bone defect. In our study, we evaluated the role of MSCs in reconstruction of bone defect in animal model.

METHODS

This prospective randomized trial was performed from December 2009 until November 2010 in the Histology laboratory and animal house, Faculty of Medicine, Universitas Indonesia. Prior to the study, ethical approval was obtained from Ethics Committee of Faculty of Medicine, Universitas Indonesia. Based on Steel and Torrie formula, twenty eight giant Flemish rabbits weighted 2.70 kg were randomly assigned into four intervention groups. Each group received a different type of graft (Autograft, hydroxyapatite-calcium sulphate [HA-CaSO₄], HA-CaSO₄ combined with marrow aspirate, or HA-CaSO₄ combined with 2x10⁶ MSCs).

Surgical procedure

Study animals were anesthetized using ketamine (35 mg per kilograms body weight). Defect of one centimeter long was created then immediately fixated with mini plate-screw. To enhance the strength of fixation, we added two extra cerclage wires.

The defects were then transplanted with the bone graft, which type is according to their allocated groups. Autograft was graft taken from the iliac wing, while marrow aspirate was aspirated from iliac marrow.

Mesenchymal stem cells preparation

Prior to surgical procedure, MSCs from seven donor Giant Flemish rabbits were isolated from the iliac crest using the same technique that was used before.⁶ The cells were seeded into 25 cm² culture flask containing low-glucose Dulbecco's Modified Eagles Medium (DMEM, Gibco, Green Island, New York) containing 10% fetal bovine serum and cultured at 37°C in 20% O₂ and 5% CO₂. Then the cells were subcultured into the first passage in 75 cm² tissue culture flask. The culture reached confluence in 8 weeks. At the time of transplantation, two pellets of hydroxyapatite-calcium sulphate (Perossal[®], GmbH, Germany) were soaked into the medium containing two millions of MSCs for one minute.

Evaluation of stem cells compatibility with hydroxyapatite-calcium sulphate

HA-CaSO₄ pellet was added into one of the cells culture. We observed the dissolution of the pellet and the growth of the cells every week under inverted microscope. After three weeks, the pellet was removed and the cell's growth was observed further for another week.

Callus thickness

The experimental animals underwent an x-ray examination every four week until twelve week post intervention. From the digital radiographs, the callus thickness at the proximal, central, and distal of the defect were measured using caliper by two independent authors who are blinded to the treatment allocation. The average of the thickness of the callus at the proximal, central, and distal of the defect defined as the callus thickness.

Osteocyte index

The experimental animals were sacrificed after 12 weeks. After the removal of the implant, the bones were decalcified and embedded in paraffin. Longitudinally oriented section of 5µm was placed on silane-coated slides and stained using tolluidine blue. The lacunae, both with and without osteocyte, were counted in three high power fields at the middle of the

defect. The percentage of the lacunae with osteocytes to those without osteocytes defined as osteocyte index.

Statistical analysis

Callus thickness was analyzed using one-way ANOVA with Tukey HSD as the post-hoc analysis. Due to the small sample size, osteocyte index could not be analyzed statistically and presented descriptively.

Ethical clearance

The ethical clearance was acquired from the Research Ethic Committee of Faculty of Medicine, Universitas Indonesia. (No. 288/PT02.FK/ETIK/2009).

RESULTS

At eight week, confluence was achieved and more than 60 million cells were yielded from each flask. In the culture medium, HA-CaSO₄ pellet dissolved into crystals (Figure 1). These crystals would be washed out by changing the culture medium and would not interfered with cells growth.

The mean weight of the animal evaluated was 2695 ± 596.84 g and the femoral length was 12.3 ± 1.3 cm. The characteristics of the evaluated animal are shown in table 1.

During our study, many animals did not survive due to diarrhea outbreak. Evaluable animals are

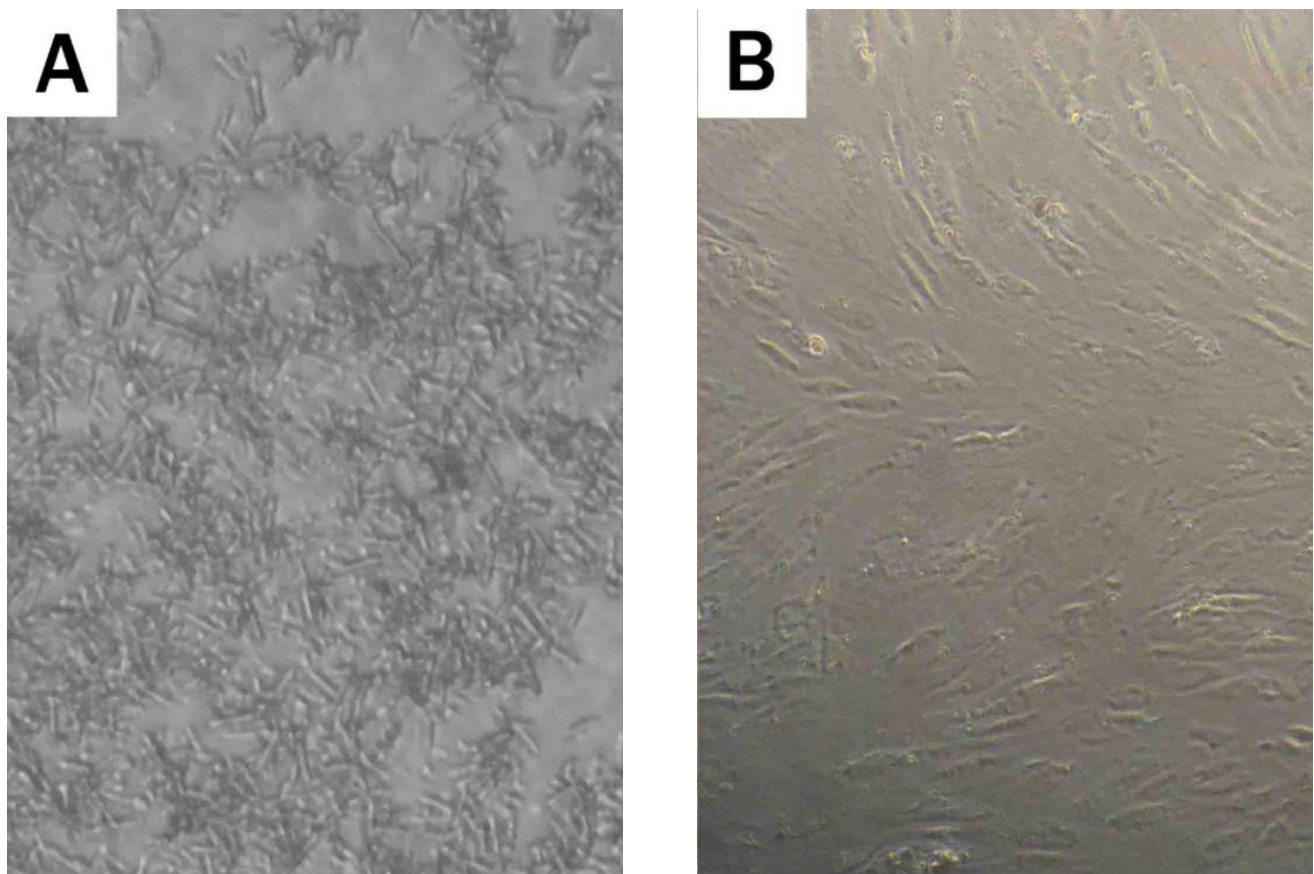


Figure 1. Hydroxyapatite-calcium sulphate dissolved in culture medium. A. During the first week, the crystals filled up the culture area and cells were hardly visible under microscope. B. At the fourth week, after the removal of the pellets, the cells were still viable

Table 1. Baseline characteristics of the animal

Characteristics	Autograft (n = 7)	HA-CaSO ₄ (n = 7)	HA-CaSO ₄ + aspirate (n = 7)	HA-CaSO ₄ + MSCs (n = 7)	p*
Body Weight (g)	2786 ± 486	2743 ± 753	2771 ± 599	2373 ± 518	0.525
Femoral anatomical length (cm)	9.1 ± 0.8	9.4 ± 0.6	9.2 ± 0.8	9.2 ± 0.6	0.833
Femoral width (mm)	6.8 ± 1.3	6.9 ± 1.4	6.5 ± 1.2	6.6 ± 1.2	0.943

HA-CaSO₄ : hydroxyapatite-calcium sulphate, MSCs: mesenchymal stem cells

described in table 2. The diarrhea occurrence was found not related to mesenchymal stem cells ($p = 1$) nor HA-CaSO₄ ($p = 0.318$).

Between four intervention groups, only callus thickness at the week 4 showed statistically significant difference (Figure 2). Figure 3 shows radiographs of animals from the MSCs group.

Osteocyte index also tended to be higher in MSCs group although we were unable to perform statistical analysis due to the lack of sample (Figure 4).

Table 2. Number of evaluable animal during 12 weeks observation period

	Week 1	Week 4	Week 8	Week 12
Autograft	7	5	4	4
HA-CaSO ₄	7	5	3	0
HA-CaSO ₄ +Aspirate	7	6	5	2
HA-CaSO ₄ + MSCs	7	5	4	3

HA-CaSO₄ : hydroxyapatite-calcium sulphate, MSCs: mesenchymal stem cell

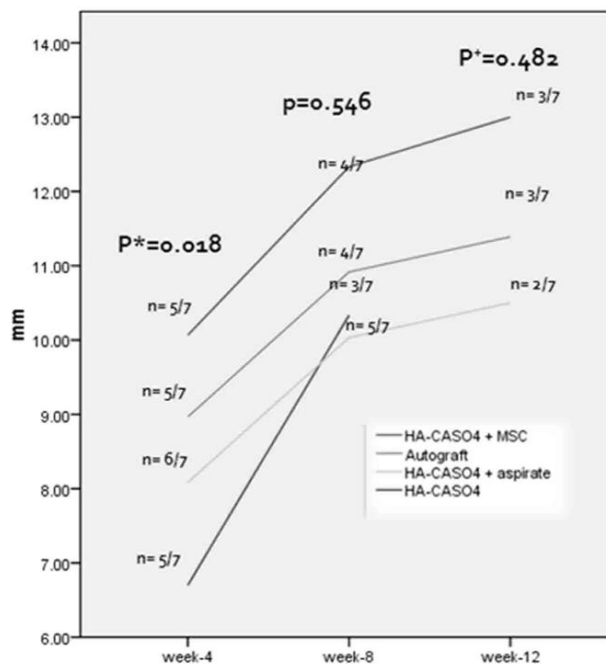


Figure 2. Callus thickness at the fourth, eighth and twelfth week. p value at week 4 and 8 was determined using ANOVA test and at week 12 using Kruskal-wallis test. The difference of the callus thickness at week 4 was due to the difference between the HA-CaSO₄ and the HA-CaSO₄ + MSCs group. (Tukey HSD $p = 0.018$)

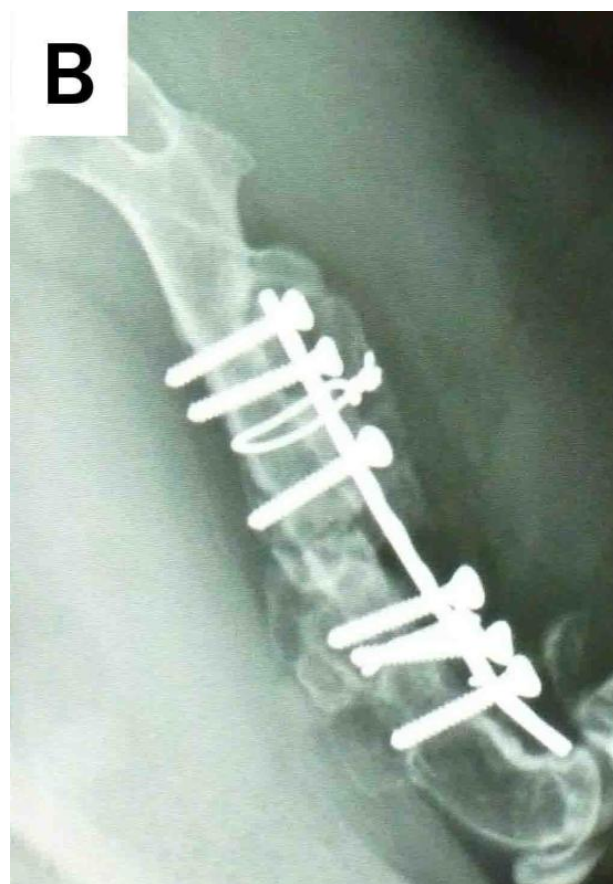


Figure 3. Defect with one centimeter of bone lost treated with combination of HA-CaSO₄ combined with MSCs. A. By the first week, no significant callus was formed. B. by the twelfth week, abundant callus was formed and fracture line was hardly visible

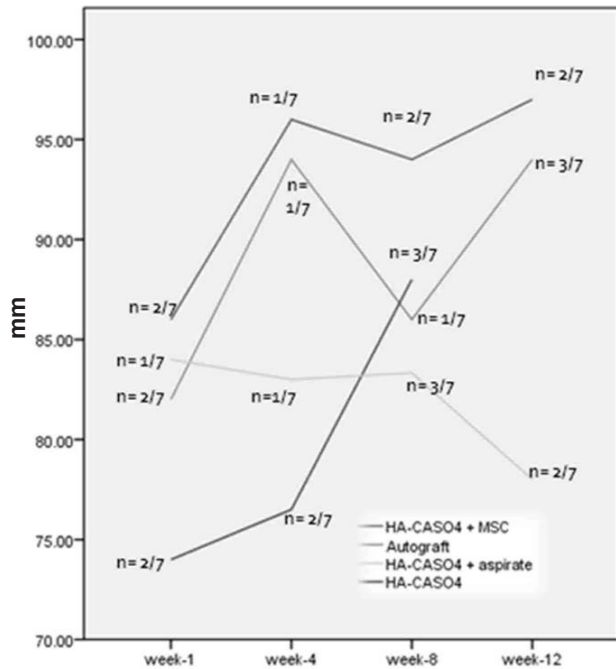


Figure 4. Osteocyte index at week 1, 4, 8 and 12. Osteocyte index tend to be highest amongst other groups at all period of evaluation

DISCUSSION

Osteoconductive scaffold is one of the components of diamond model in fracture healing, especially in bone defect.⁷ Hydroxyapatite, a crystalline scaffold, is seldom used alone due to its slow resorption and slow bone formation.⁸ Calcium sulphate, being rapidly resorbed, had been advocated by some authors to be used as osteoconductive scaffold.⁹ However, a study reported that calcium sulphate, if used alone, would have showed some cytotoxic effects.¹⁰ We therefore used combination of the hydroxyapatite and calcium sulphate as the scaffold. The combination had a specific surface of 106 m²/g and had been reported to show no significant *in vitro* cytotoxic differences compared to nontoxic cell culture medium.¹⁰ Our result at the first phase of the study confirmed this report.

It took longer time to achieve confluence in our study compared to other studies. Medium type and seeding density had been reported to influence MSCs growth, although the best medium type and seeding density remain a controversy.^{11,12} Rapid expansion after initial seeding was reported in a commercial serum-free culture medium, however it failed to maintain the rate after subsequent passages.¹³ Refreshing only a part of the culture medium was also recommended by some authors.¹¹

Although not addressed in our study, long term culture of MSCs might subject to Hayflick limit.¹⁴ The

proliferation rate decayed, the cell sized increased, and the differentiation potential was affected, chromosomal instabilities arose, and molecular changed were acquired. The malignant transformation of long cultured MSCs remains a controversy and need further exploration.^{15,16}

We compared the potency of MSCs with several other methods of treatment of bone lost, including autograft as the gold standard. In order to avoid bias, we also evaluated and compared the result with transplantation of the scaffold alone, the HA-CaSO₄, and also HA-CaSO₄ in combination with marrow aspirate.

Diarrhea outbreaks caused many death of the animal since the fourth week. Since the outbreaks occurred in every intervention group, we could not attribute the diarrhea to occur due to specific kind of cause.

We used callus thickness as the outcome in the present study. Callus growth is an indicator of healing process and have been used to quantify fracture healing.¹⁷ The result was confirmed further with histological evaluation using osteocyte index. Osteocyte index resembles cellular activity.¹⁸ The higher the cellular activity, the more likely the graft will survive.

Callus formation started at the first week of intervention. However, the formation was unreliable as indicated by considerable standard deviation. They also varied insignificantly between groups of intervention. This finding is relevant to the first healing phase of fracture.¹⁹ The first phase is marked by the hematoma formation and the resorption of the bone. No callus formation was found during this phase.

The second phase is the callus formation.¹⁹ Callus is initially liquid in consistency and slowly solidify and become readily visible in radiological imaging. This phase is in accordance to our finding at the fourth week. Reliable callus had been formed, particularly in the MSCs group. This finding can be explained from the fact that although osteoblast cells are readily available in bone, the new bone formation (callus) is mediated from the differentiation of progenitor cells which formed new osteoblast that are in charge for the callus formation.²⁰

By the eight week, no significant difference was found between the intervention groups statistically. At this period, we believed that the amount of new osteoblast cells that have been formed is quite enough in every intervention groups. Although statistically insignificant, callus in MSCs group seemed to be thicker than any other groups of intervention.

At the twelfth week, only three groups of intervention could be analyzed. We noticed that callus thickness in the MSCS group was thinner than the thickness at eighth week. We proposed that remodeling phase might have taken place.

Although our result shows promising role of MSCs, it should be noticed that our sample size was not adequate especially in the twelfth week. Most of the samples were injured by the diarrhea outbreak. Since the measurement of callus thickness is subjective, it was measured by two independent authors.

In summary, we conclude that MSC transplantation on bone defect results significantly faster callus formation and tend to generate a thicker callus.

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Conflict of interest

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