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Repair of Compact Bone Critical Sized Defect with Natural Originated Scaffold in Rabbit

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Abstract: The main aim of the present study was to investigate, the effect of combination of bone marrow as the primary origin of osteoblast and at the same time as the seed cell and corticocancellous bone graft as the natural scaffold in the repair of critical sized defect compact bone in rabbit. For the test group, bone marrow has been aspirated and seeded into the corticocancellous bone graft, which was used to repair critical size bone defect made in mid shaft femoral bone of the same rabbit. Corticocancellous bone graft itself was utilized as the control group. Radiographs were taken to observe the healing during the 8 weeks of study period. Rabbits were euthanized after 8 weeks. The femoral bone was removed for gross observation, histopathological and scanning electron microscope assessment and evaluation. New bone formation and osteogenesis was observed at the margins and centre of the test group. The bone formation pattern included osteogenesis, osteoinduction and osteocunduction. In the implant of only corticocancellous autograft bone, the major new bone formation was at the margins of the defect and osteogenesis was not observed at the centre of the defect. The combination of bone marrow and corticocancellous bone autograft had better results than corticocancellous bone graft alone in osteogenesis. Bone formation capability and critical sized defect repair was faster in the test defect.

Key words: Compact bone, critical sized defect, bone marrow, corticocancellous bone graft, scaffold mesenchymal stem cell and osteobast

INTRODUCTION

The natural processes of bone repair are sufficient to effect timely restoration of skeletal integrity for most fractures when, an appropriate mechanical environment exists or is created with internal fixation or external coaptation. However, some situations require manipulation or augmentation of natural healing mechanisms to regenerate larger quantities of new bone than would naturally occur to achieve surgical goals. Specific complications that may require additional interventions include substantial loss of host bone due to trauma or tumor resection, non-union or delayed unions, metabolic disease, insufficient healing potential of the host bone because of local or systemic disease, osteogenesis imperfecta, osteoporosis and old age. Materials and strategies that are employed must duplicate and amplify the events of secondary bone union to achieve the desired result (Attawia *et al*., 2003; Cancedda *et al*., 2003).

Through previous years, many effort have been made to investigate different techniques and material inductive to rapid flawless osteogenesis and thus, avoiding mentioned complications and side effects and also regaining normal physiologic and anatomic abilities of the affected bone. It is now believed that filling the proposed defective site with some bone substitutions possessing osteogenic potentials is the main key to this goal hat is the completely healed bone with normal anatomic structure and function. Variety of materials including biosynthetic materials as well as natural substances have been tested that can help the formation of new bone through the following categories; osteogenesis the transfer of cells; osteoinduction the induction of cells to become bone; osteoconduction, providing a suitable scaffold for bone forming cells and osteopromotion the promotion of bone healing and regeneration by encouraging the biologic or mechanical environment of the healing or regenerating tissues (Attawia *et al*., 2003; Moore *et al*., 2001).

Among the materials qualified to substitute the bone natural substitutions such as bone graft are preferred to biosynthetic structures because, even very negligible they provide a good source of osteogenesis and

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osteoinduction potentials. Autogenous bone graft is considered as the gold standard graft (Bonye *et al*., 2002; Zhi and Zu-Bing, 2005; Parikh, 2002) with the highest capability of osteoinductivity in comparison to the allograft and xenograft, which they usually prevent complete bone ingrowth due to short of osteogenetic cells (Zhi and Zu-Bing, 2005; Krzymanski *et al*., 1997). Each of the grafts can play a role as a scaffold to let the bone regenerate itself. However, the characteristic of absorption, biodegradation and amount of osteogenetic cells of these materials are such different that can influence the quality and quantity of new formed bone.

Cancellous bone autograft has the highest power of osteogenecity among the grafts; but harvesting this graft accompanies with some inevitable disadvantages such as morbidity and pain of the donor site or limited supply. Corticocancellous bone autograft besides of possessing some characteristics of cancellous bone autograft, is not carrying its ghastly side effect, which is related to its way of harvesting. The osteogenecity power of corticocancellous bone autograft can become further if it is seeded with another ostegenic substitution such as mesenchymal stem cell or osteoblasts. To over come the cumbersome process isolation of mesenchymal stem cell and its differentiation into osteoblast and also to find out whether the bone marrow itself can act as its progenies (mesennchymal stem cell and osteoblast), bone marrow have been utilized in the current study.

The primary purpose of the present study was to observe the effect of combination of corticocancellous bone autograft as the scaffold seeded with bone marrow as the origin of mesenchymal stem cell, which has the capability to differentiate into osteoblast and osteocyte, respectively in repairing the critical sized defect in compact bone.

MATERIALS AND METHODS

Compact bone defect model preparation and repair: Twelve New Zealand white rabbits, 5 months old of both sexes (average weight 2.5 kg) ware chosen for the present study. The protocol for this research project and animal experiment in this study has been approved by Animal Care and Use Committee of Faculty of Veterinary Medicine of University Putra Malaysia (No.: 08R10).

The animals were anesthetized using intramuscular injection of mixture of ketamine (100 mg mL^{-1} and 40 mg kg^{-1}), xylazine (20 mg mL^{-1}; 5 mg kg^{-1}) and acepromazine (5 mg mL^{-1}; 1 mg kg^{-1}). Surgery was performed under sterile condition. Femoral bone was exposed through the craniolateral approach. Mid shaft point of femur was determined measuring the distance between greater trochanter and patellar level. Ten mm full thickness defect was created (5 mm above and below the mid shaft point) using gigli saw. Rectangular bony defect of 10×5 mm is a documented as critical sized defect in the rabbit (Zhi and Zu-Bing, 2005). The fracture was fixed using bone plate 2 mm. Half of the rabbits in group one received corticocancellous bone graft as the control group and the others in group two received combination of corticocancellous bone autograft and fresh bone marrow as the test group. All animals were given IM injection of penicilline (400000 IU mL^{-1}; 0.1 mL/kg/day) in the first 3 days post operation as well as tramadole (50 mg mL^{-1}; 4 mg kg^{-1}) as the analgesic preemptively and a day post operative.

Corticocancellous bone graft harvest: Under sterile condition, wing of ilium was exposed using cranial dorsal iliac spine approach. About 15 cm^2 of corticocancellous bone graft was harvest from wing of ilium by rounger and then it was cut into pieces of 0.2×0.2×0.1 mm. Pieces of corticocancellous bone graft were placed in the fracture gap between the proximal and distal fragments to fill the gap completely in group 1.

Bone marrow harvest and seed into corticocanceelous bone graft: Two mL of Bone marrow was aspirated from wing of ilium by J style bone marrow aspiration needle. It was then transferred into the EDTA tubes to be prevented from coagulating. Bone marrow then was seeded into harvested corticocancellous bone autograft chips by placing the bone graft pieces into the tube. It was left undisturbed for 10 min to allow the bone marrow cells to attach. The bone marrow seeded corticocancellous bone autograft pieces were placed in the fracture gap between proximal and distal fragments to fill the gap completely in group 2.

Specimen examination: The femur was radiographed preoperative, post operative and once a week after surgery up to 8 weeks. After 8 weeks the rabbits were euthanized by intra cardiac injection of dolethale (1 mg kg^{-1}). The femur bone was removed for gross observation, histological assessment and Scanning Electron Microscope (SEM) evaluation. Statistical evaluation was made by kruskal-wallis, nonparametric analysis using SPSS 16.0 version, $p<0.05$ was considered as statistically significant.

RESULTS

Radiographic observation: Radiographs were taken every week using table top procedure (47 kVp and 2 mAs) with exposure time of 0.04 sec and a working distance of 1 m.

Primary sign of callus formation was fashioned 1 week postoperative in test group whereas spots of callus was seen at 3rd week post operation in control group. At the 3rd week, callus occupied the whole defect in test group, while, same thing happened in the control group in 5th week. On the control defect the callus was mainly formed at the margins near the host bone. In the test group at 4th week, osteogenesis was obvious at defect margins but it was not detectable up to week 6 in the control group. At the 8th week, the defect margins were not visible and the defect was completely filled with new bone in the test group. Also the bone density was similar to the host bone (Fig. 1-2). In control group at the same time, new bone formation was seen at the defect margins but centre of the defect was not filled with new bone and there was a radiolucent region remained at the center of the defect indicating incomplete healing (Fig. 3-4).

Gross observation: No animal died during the postoperative period. Healing after the operation progressed uneventfully. After euthanasia femur of both groups was removed and assessed. In test group, the bone plate was in place and bone was completely healed. There was neither excessive callus formation nor any adhesion between bone and surrounding muscles (Fig. 5). In contrast, in control group although, the bone plate was intact, there was abundant callus formed, which had embedded the bone plate (Fig. 6). New bone has started to grow from the proximal and distal fragments but they didn't meet at the center of the gap. On the other hand, central part of the control defect did not have the bony structure and strength. Also, there was adhesion between callus and surrounding soft tissue.

Histological observation: Microscopic examination showed normal bone morphology in both experiment and control implants; no inflammatory response was observed.

After euthanasia at 8th week the sample has been taken for histopathology assessment. The sample was taken from 2 mm proximal to defect to 2 mm distal to the defect in a way the healing as well as the connection between new formed bone and old bone could be observed in one shot. Samples were stained using H and E technique. In the test defect at the 8 weeks, newly formed bone was observed (Fig. 7). The corticocancellous bone graft was completely resorbed, there was connection between new bone and old bone some how that distinguishing them from each other was very difficult. There was no difference between new bone and old bone. Periosteum had normal configuration and was completely attached to new bone (Fig. 8). In control defect

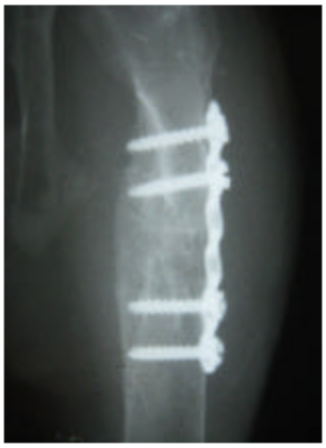

Fig. 1: CrCd radigraph view of test group at week 8 shows complete healing

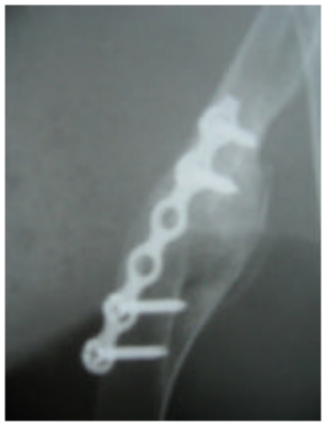

Fig. 2: L at radigraph view of test group at week 8 shows complete healing

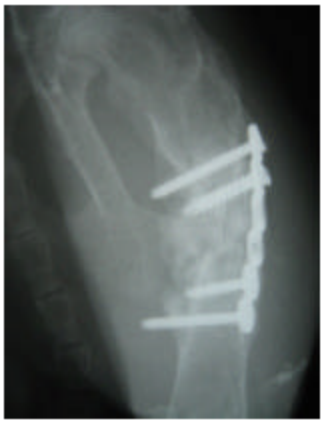

Fig. 3: CrCd radiograph view of control group at week 8 shows radiolucent dencity at the centre of the defect

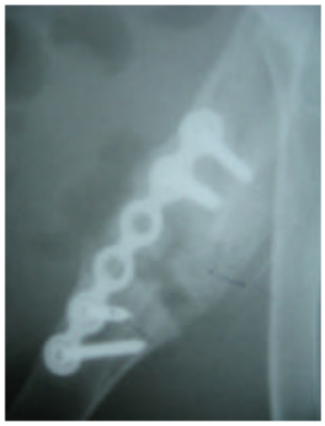

Fig. 4: Lat radiograph view of control group at week 8 shows not a bone density at the middle of the defect

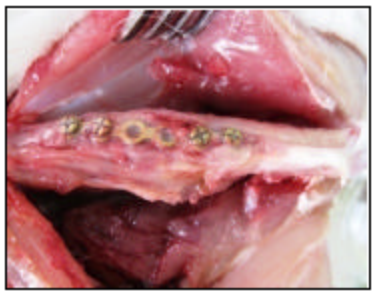

Fig. 5: In test group, intact bone plate, not excessive callus, normal shape of the bone and no bone-soft tissue adhesion in obvious

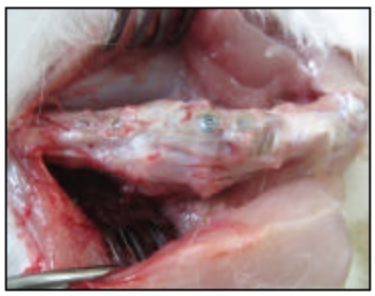

Fig. 6: In control group abundant of callus formation, bone plate embedding by soft tissue and lots of bone-soft tissue adhesion was the findings

Gross observation: No animal died during the postoperative period. Healing after the operation progressed uneventfully. After euthanasia femur of both groups was removed and assessed. In test group, the bone plate was in place and bone was completely healed. There was neither excessive callus formation nor any adhesion between bone and surrounding muscles (Fig. 5). In contrast, in control group although, the bone plate was intact, there was abundant callus formed, which had embedded the bone plate (Fig. 6). New bone has started to grow from the proximal and distal fragments but they didn't meet at the center of the gap. On the other hand, central part of the control defect did not have the bony structure and strength. Also, there was adhesion between callus and surrounding soft tissue.

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Since, we had harvested the corticocancellous bone autograft as the scaffold from each animal at the surgery time, to assess the healing of the wing of ilium, the samples of this bone at the harvesting site were evaluated histopathologicaly. In both control and test defects, the iliac bone have been healed completely and all lacunas were filled with the osteocytes, which indicate live bone. This assessment implies that corticocancellous have fewer side effects than cancellous bone graft.

Electron microscope observation: Followed by light microscope, the defect was scanned by scanning electron microscope. In test defect at the 8th week, flat, dense and smooth surface with abundant of osteocytes in lacuna

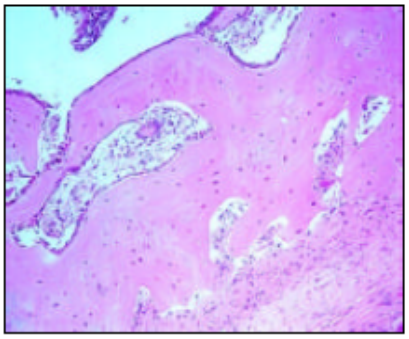

Fig. 7: At week 8 in test group, immature bone with high osteoblast appositional surface and normal periosteal configuration was seen

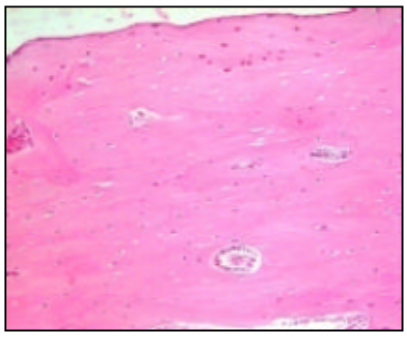

Fig. 8: Newly formed bone with osteoblast attached to the surface was observed

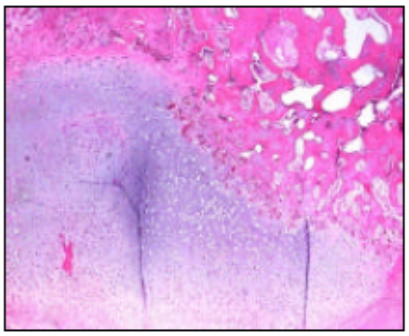

Fig. 9: In the control group at week 8 new bone formations was not seen at the middle of the defect. Hypertrophied chondrocytes at the centre of the defect surrounded by new bone at the periphery was the findings

that filled the entire defect was seen (Fig. 11), while in control defect at the same time uneven and non flat surface with porous view and with not much amount of osteocytes was observed, indicating incomplete healing (Fig. 12).

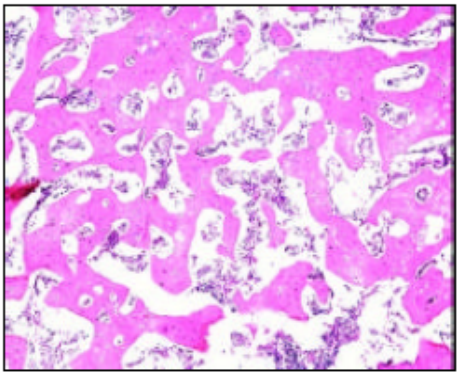

Fig. 10: Major pattern at 8th week in control group was creeping substitution

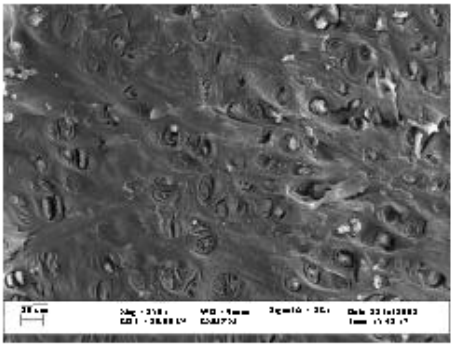

Fig. 11: Abundant of lacuna filled with osteocytes in dense extra cellular substances was seen under SEM in test defect at 8th week

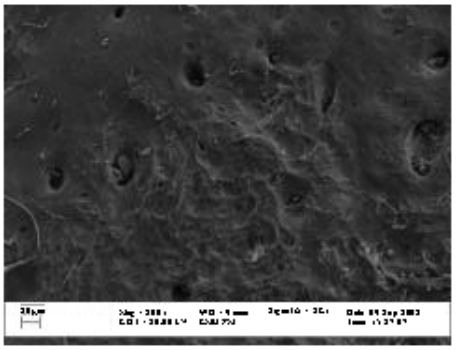

Fig. 12: At 8th week in the control group, few numbers of osteocytes have seen at the defect margins

DISCUSSION

The result of our study gives the impression to indicate that both the test and control defects could succeed in repairing the compact bone defect. However,

the combinations of corticocancellous bone autograft and bone marrow resulted in higher quality and quantity of new bone formation than corticocancellous bone graft alone. Also, the healing time in these 2 groups is significantly different.

Radiographic images presented callus as well as new bone formation primarily at the margins of the defect and also gradually it was pulled towards the center of defect in test group, which indicates osteogenesis at both margin and centre of defect during the healing period. However, the newly formed bone in corticocan cellous bone autograft alone was mainly visible at the margins of the defect and the callus formed more slowly than the test group. It gives the impression that the new bone formation of corticocancellous bone autograft alone depended on the ingrowth of the new bone from the host bone (Zhi and Zu-Bing, 2005). By histopathology examination, in test defect, the bone formation was observed at both margins and centre of the defect, which approves the radiographic findings. There were large amount of trabecula penetrated into the implant and the remodeling and mineralization of new formed bone occurred more quickly. However, in control implant, the bone formation was not completed at the centre of the defect as it was observed in the radiographs. The bone formation pattern was mainly creeping substitute and there was very little and slow osteogenesis at the centre of the defect. With scanning electron microscope the findings of radiography as well as histopathology was confirmed. As shown, the defect in control and test groups have distinct differences with each other, indicating complete healing with dense surface in test defect but incomplete healing with porous view in control defect. Dense extracellualr matrix with lacuna filled with osteocytes all over the defect proofs the complete healing in test group as observed under SEM. These data together suggest that osteogenesis, osteoinduction and osteoconduction, which are necessary for a good quality and fast healing-could simultaneously occur in the test defect but, the osteoconduction and very little osteoinduction (because of the osteoinductivity nature of corticocancellous bone graft) was the main pattern in new bone formation in control defect.

Recently, bone tissue engineering, which is combination of scaffold and a seed cell has been heralded as the strategy to regenerate bone because, it can provide adequate bone volume and satisfactory bone regeneration potential. A central tissue engineering approach is the *in vivo* implantation of a biosynthetic or natural scaffold seeded with an appropriate population of osteogenic and osteoinductive seed cells (Rose and Oreffo, 2002). We believe that the marrow enhanced the bone formation due to the presence of mesenchymal stem cell that could be differentiated into osteoblast.

An important boundary condition for bone tissue engineering is to obtain the seed cells. Bone marrow has been widely seeded in different scaffolds. Bone marrow contains osteoprogenitor cells and induces bone formation both *in vivo* and *in vitro* (Wang *et al*., 2003).

Mesenchymal stem cells are multipotent cells present in a variety of tissues during development and in adults mainly in bone marrow. Bone marrow mesenchymal stem cells may be isolated and expanded *in vitro* and they are capable of differentiating into a variety of tissue, including bone, cartilage, muscle and adipose tissue.

Scaffold is another key element effective in good and fast bone regeneration. An ideal scaffold should be: three dimensional and highly porous with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste; biocompatible and bioresorbable with a controllable degradation or resorption rate to match cell/tissue growth *in vitro* and *in vivo*; suitable surface chemistry for cell attachment proliferation and differentiation and capable of osteoinduction and osteoconduction (Hutmacher *et al*., 2003; Burg *et al*., 2000).

Corticocancellous bone autograf has good biocompatibility, suitable surface chemistry and 3-D porous network system and it can degrade *in vivo*. The osteoinductive property of corticocancellous bone autograt has stimulated its wide use in compact bone augmentation and reconstruction.

Altogether, these observations indicate that the combination of autogenous corticocancellous bone graft and bone marrow has the capability of osteogenesis, osteoinduction and osteoconduction with a better osteogenetic effect and quality than corticocancellous bone graft implant alone. Due to the availability of bone marrow and also the easy harvesting technique of corticocancellous bone besides not carrying the cancellous bone autograft disadvantageous, along with the bone regeneration potential, it might be an ideal graft for bone defect repair in hospitals. However, what we found in this research is that the combination of corticocancellous bone graft and bone marrow resulted in

a higher quality and quantity of new bone formation than the implant of corticocancellous bone graft alone at the same period within 8 weeks. These results cannot absolutely exclude the possibility that the corticocancellous bone alone gives the same result by 16 or 20 weeks.

In this research to overcome the costly, time consuming and cumbersome stages of *in vitro* isolation, proliferation and differentiation mesenchymal stem cell into osteoblast, the bone marrow itself has been seeded into the scaffold. It was observed that without *in vitro* differentiation of osteoblasts, bone marrow can also been used as an osteogenic and osteoinductive feature and accelerate the healing. Now there is a challenging question raise here; while, the bone marrow has the osteogenecity and osteoinductivity potential of osteoblast, is the cost, time and effort effective for isolation, proliferation and differentiation of mesenchymal stem cell into osteoblast worthwhile to expend? This is an interesting question needed further investigation due to importance of practical application of bone marrow and osteoblast in bone healing in clinical cases.

CONCLUSION

Osteoblast could be differentiated from bone marrow mesenchymal stem cell *in vitro* and can be seeded in different natural or synthetic scaffolds. In this study, the bone marrow itself as the primary origin of osteoblast has been seeded in the cancellous bone graft as the natural scaffold and showed extraordinary results in critical sized defect repairing.

Thus, it might be an ideal graft in bone losses and when, there is need of excessive ostegenecity potential such as delayed and nonunions.

ACKNOWLEDGEMENT

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