

Bone Marrow Seeded Bone Graft Versus Bone Graft; Compact Bone Critical Sized Defect Healing Pattern 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 repair of compact bone full thickness segmental critical sized defect in rabbits. Twelve rabbits had been divided into two groups; In Group one, fresh autogenous bone marrow aspirate has been seeded into the scaffold of autogenous corticocancellous bone graft which was utilized to repair critical size compact bone defect in mid shaft of radius. Corticocancellous bone graft alone was used as the Group 2 or control group. Up to 8 weeks, radiographs were taken to evaluate the level of osteogenicity in both groups. Rabbits were euthanized on week eight postoperative and the implants were harvested for gross, histological and scanning electron microscope observations. New bone formation and osteogenesis was observed at the margins and centre of the Group 1. Combination of mature and immature trabeculae covered the defect and bone formation pattern included osteogenesis, osteoinduction and osteoconduction. In the implant of corticocancellous bone graft alone or group 2, the major new bone formation was at the margins of the defect and osteogenesis was not observed at the centre of the defect and the major bone formation pattern was creeping substitution. As the conclusion, combination of bone marrow and corticocancellous bone graft had better results than corticocancellous bone graft alone in osteogenesis potential. Bone formation capability and critical sized defect repair was faster and more efficient and successful in Group 2 defect.

Key words: Bone marrow, bone graft, critical size defect, mesenchymal stem cell, bone tissue engineering, rabbit

INTRODUCTION

Restitution of skeletal anatomical and physiological impeccability as well as new bone tissue regeneration is still an imperative challenge. Occasions that require further efforts to restore this integrity include Augmentation of fracture healing, treatment of mal, non and delayed unions, reconstruction of bone defects, trauma, tumor, infections, corrective osteotomies, bone disease such as osteoporotic fractures and osteogenesis imperfecta. In such cases besides surgically reduction and fixation of the affected bone, other endeavors should be considered and attempted.

Different bone grafts of various natures have been used to augment orthopedic defects in veterinary as well as human surgery for several decades and still researchers are looking into new approaches to improve bone healing pattern in health and diseases (Fox, 1984; Griffon, 2002).

Augmentation of bone healing by bone autogenous, allogenic grafting application is used in significant numbers of orthopedic surgeries each year. Also, different types of bone graft substitutes have been introduced to the grafting list in the recent years.

In addition to utilizing these graft materials per se, they could also be seeded with osteogenic and/or osteoinductive elements to augment and boost the genicity and inductivity potentials and efficacies. Some of these elements include osteogenic lineage cells such as Mesenchymal Stem Cells (MSC), Bone Marrow (BM) that carries MSC and Osteoblast (OSTB) or osteoinductive growth factors such as Bone Morphogenic Proteins (BMP), Fibroblast Growth Factor (FGF) and Transforming Growth Factor (TGF). Natural and synthetic grafts and grafts substitute can play a role as scaffold to carry these elements into the defect site. Seeding these elements on grafts and graft substitute not only boost their

osteogenic/inductive potential but also may decrease the utilizing quantity of the grafts. There are four characteristic features that an ideal graft material should exhibit which include: osteogenesis, osteoinduction, osteoconduction and osteointegration (Moore *et al.*, 2001). Only autogenous bone graft satisfies all of these requirements.

Allografts are osteoconductive and may only in some cases (such as demineralized bone matrix) exhibit osteoinductive potential but it does not carry osteogenic properties given that it contains no live cellular component. Synthetic bone graft substitutes possess only osteoconductive properties but not genic and inductive ones.

Fresh autogenous bone graft is deemed as the most efficient grafting material since it provides the highest number of viable osteoprogenitor cells and contains noncollagenous matrix protein and growth factors as the osteoinduction. It also carries bone mineral and collagen which provide a scaffold for osteoconduction means (Keating and McQueen, 2001; Ladd, 1999; Linovitz, 2002). It neither initiates or increases immunologic reaction, host rejection nor transmits diseases as seen in allografts. Despite bone graft substitutes that have little biological activity autologous graft has a considerable biological activity (Moore *et al.*, 2001). These synthetic bone grafts mostly have only conductive properties.

Bone marrow is other constituent of autogenous grafting material. It has three different class of stem cells including Mesenchymal Stem Cell (MSC), Hematopoietic Stem Cell (HSC) and epithelial stem cell. MSC are the osteoprogenitor stem cell, capable of bone forming when combined with various elements of an osseous matrix (Millis and Martinez, 1993). Large osseous defects can be successfully healed when fresh or cultured BM which is added to the defects. Indication of use of fresh bone marrow is the same of that of cancellous bone autograft (Millis and Martinez, 1993).

Osteoblastic stem cells are found in bone marrow. Grafting of autologous bone marrow can supply a graft that is osteogenic and potentially osteoinductive through cytokines and growth factors secreted by the grafted cells. Bone marrow can be aspirated from the anterior and posterior iliac wing and can be grafted into a fracture or nonunion site to stimulate and boost the healing (Finkemeier, 2002). Bone marrow can be grafted either alone or seeded on scaffolds. Percutaneous injection of bone marrow is a routine and safe technique for the bone marrow grafting alone. Bone marrow also could be seeded on different natural or synthetic scaffold before grafting and graft then is comprise of scaffold seeded bone marrow and is being reported to be more efficient that

both BM and scaffold alone; however is completely depended on the number of progenitor cells of grafted and seeded BM and properties (genic, inductive or conductive) of scaffold. So far even with over emphasized donor site morbidity shortcomings of autograft, this graft material is still the gold standard and all other grafts and graft substitutes, either seeded or non-seed are compared with this type of graft in their success rate in new bone regeneration potential and efficacy (Healy and Guldberg, 2007).

In recent years, there has been considerable improvement in understanding bone repair utilizing bone tissue engineering approaches which is *in vivo* implantation of a suitable scaffold seeded with appropriate population of seed cells and/or growth factor at the bone defect site (Bone tissue engineering; <http://www.btec.cmu.edu>). This has significant implications on the management of bone losses. Bone tissue engineering has opened a new window to evaluate the potential of different scaffold seeded with osteogenic lineages on new bone regeneration.

The main aim of the current research was to investigate amount of new bone regeneration of autogenous bone graft seeded with autogenous fresh bone marrow aspirate on bone defects. This combination due to osteogenic, inductive and conductive potentials of both scaffold and seeds is considered the one the most potent graft material thus far used to treat osseous defect requires further attempts besides surgical intervention.

MATERIALS AND METHODS

Animals and design: Twelve New Zealand white rabbits, 5 months old of both sexes (average weight 2.5 kg) were chosen for the present study. The protocol of 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. Rabbits were divided randomly into two groups ($n = 6$); Group 1 or experiment group was treated with fresh autologous bone marrow aspirate (the seed cells) seeded scaffold of corticocancellous and Group 2 or control group, received corticocancellous bone graft alone (scaffold).

Anesthetic protocol: The animals were anesthetized using intramuscular injection of premixed combination of ketamine (100 mg mL^{-1} ; 40 mg kg^{-1}), xylazine (20 mg mL^{-1} ; 5 mg kg^{-1}) and acepromazine (5 mg mL^{-1} ; 1 mg kg^{-1}). About 1% isoflurane in 0.6 oxygen flow rate in completely open system using Y piece was utilized as maintenance. Tramadole (50 mg mL^{-1} ; 4 mg kg^{-1}) injected

as analgesic medication, preemptively and a day after surgery. All animals were given I.M injection of penicilline ($400000 \text{ IU mL}^{-1}$; $0.1 \text{ mL/kg day}^{-1}$) in the first three days post operation as a anti microbial treatment.

Surgery protocol

Compact bone critical size defect model preparation and repair: Surgery was performed under complete sterile condition. The right radius was exposed through medial approach. Mid shaft point of radius was determined by measuring the distance between radial head and the styloid process. The 15 mm full thickness segmental Critical Sized Defect (CSD) (Peifu *et al.*, 2008) was created (7.5 mm above and below the mid shaft point) using a fine saw. For this matter, following skin incision and subcutaneous fascia undermining, the deep antebrachial fascia was incised between the extensor carpi radialis and pronator muscles with proximally paralleling the incision to the extensor carpi radialis. While the mid shaft of radius was reached CSD was created. Rabbits were treated based on their groupings. The CSD was not fixed by any internal or external fixation. Since the radius and ulna are adhered together tightly with interosseous membrane, adequate stability was achieved after osteotomy by leaving the ulna intact without any fixation of the radius (Emami *et al.*, 2002). For the last step, muscles and skin incision were sutured.

Corticocancellous bone graft harvest: Under sterile condition, the wing of ilium was exposed using cranial dorsal iliac spine approach. Following skin and subcutaneous incisions, an incision was made on the periosteal origin of middle gluteal muscle on the lateral edge of the ilium near the cranial dorsal iliac spine and continued to beyond the caudal dorsal spine. The middle gluteal muscle was elevated caudally and continued to the caudal dorsal iliac spin. By exposing the iliac wing entirely, adequate amount of bone graft (1.5 cm^2) was harvested by rongeurs and was cut into graft chips with pieces dimension of $2 \times 2 \times 1 \text{ mm}$. The incision was sutured following graft harvest.

Bone marrow harvest and seeding into corticocancellous bone graft: About 2 mL of Bone marrow was aspirated from wing of ilium by 18 g bone marrow aspiration needle prior to bone graft harvest. It was then transferred into the EDTA tubes to be prevented from clotting. Bone marrow then was seeded into harvested corticocancellous bone autograft chips by placing the bone graft pieces into the tube containing bone marrow. It was then left undisturbed for 15 min to allow the bone marrow cells to attach into the scaffold. 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 and data analysis: The radius 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 radial bone was then 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 AND DISCUSSION

Radiographic observation: Radiographs were taken every week using table top procedure by 44 kVp, 2 mAs with an exposure time of 10 ms and a working distance of 1 m. Primary sign of callus formation was fashioned 2nd week postoperative in Group 1 whereas spots of callus was observed at 3rd week post operation in Group 2. At the 4th week, callus occupied the whole defect in first group while same event happened in the second group in 5th week post operation.

In Group 1 at 6th week, osteogenesis was obvious at defect margins but it was not detectable up to week 8 in the control group. At the last week or week 8 in group 1, the defect margins were hardly detectable and the defect was completely filled with same bone density entirely. Also the bone density was similar to the host bone (Fig. 1). In the control group at the same time, new bone



Fig. 1: Lat. view, Group 1 day 56. Bony density visible through the defect. Entire defect shows same density that is bone density



Fig. 2: Lat. view, Group 2, day 56. Margins have completely higher density compared with defect centre which is closer density to the host bone than the centre indicating bone formation at the margins and cartilage at the centre of the defect

formation was mainly observed at the defect margins but centre of the defect was not filled with bone tissue density and there was a radiolucent region with cartilaginous density remained at the center of the defect indicating incomplete healing (Fig. 2).

Gross observation: No animal died during the postoperative period. Healing after the operation progressed uneventfully. After euthanasia radius of both groups was removed and assessed. In Group 1, defect showed same appearance entirely through the defect. There was neither excessive callus formation nor so much adhesion between bone and surrounding muscles. Medullary cavity was filled with bone trabeculae completely however, there was not sign of marrow cavity or bone marrow formation (Fig. 3).

In contrast in Group 2, there was abundant of callus formation. Defect margins had bone appearance while defect centre manifested whitish cartilage look. 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 word, central part of the control defect did not have the bony structure and strength. In the medullary cavity, the margins were occupied by bone trabeculae while the centre had a cartilage view. Also there was a considerable adhesion between defect and surrounding soft tissue (Fig. 4).

Histological observation: Microscopic examination showed normal bone morphology in both experiment and



Fig. 3: Group 1 week 8. Host bone and defect almost have same color and appearance indicating both are from same tissue



Fig. 4: Group 2 week 8. Completely two distinct region is recognizable. Middle of the defect with cartilaginous shape and margins with bone appearance are clear. Huge amount of callus formation is seen

control implants; no inflammatory response or cells were observed. After euthanasia at 8th week the sample has been taken for histopathology assesment. The sample was taken from 1 mm proximal to defect to 1 mm distal to the defect in a way that the healing as well as the connection between new formed bone and old bone could be observed in one shot. Samples were stained using routine H and E technique. In Group 1 at the 8 weeks newly formed bone was observed at both margins and centre of the defect. Defect was completely covered by combination of mature and immature bone trabeculae. By

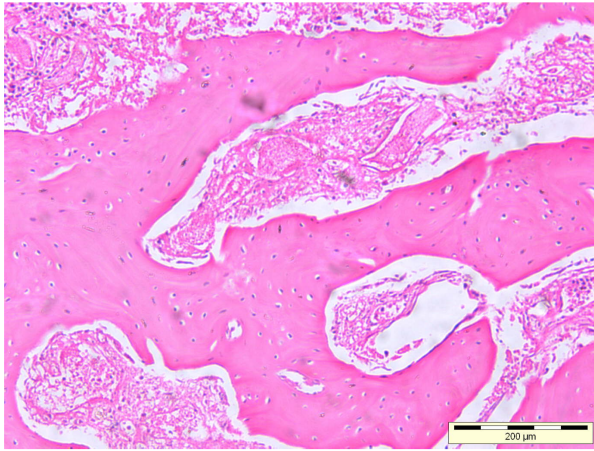


Fig. 5: Group 1 week 8. Mature marginal trabeculae (x10)

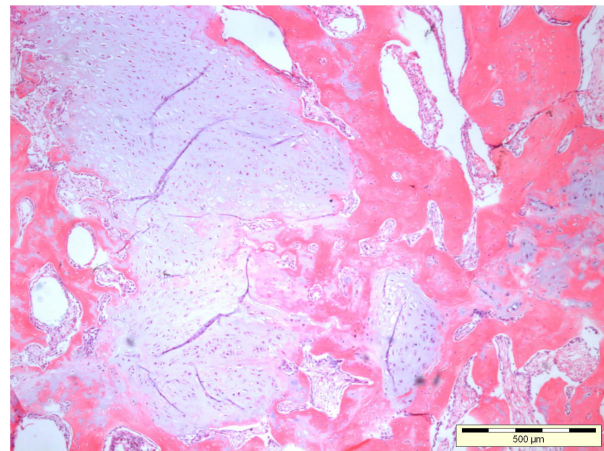


Fig. 7: Group 2 week 8. Defect center filled with hypertrophied chondrocytes and margins with immature trabeculae (x4)

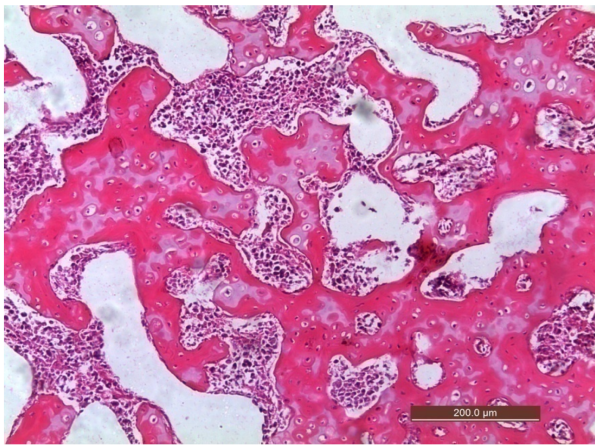


Fig. 6: Group 1 week 8. Defect centre was completely filled with immature trabeculae (x10)

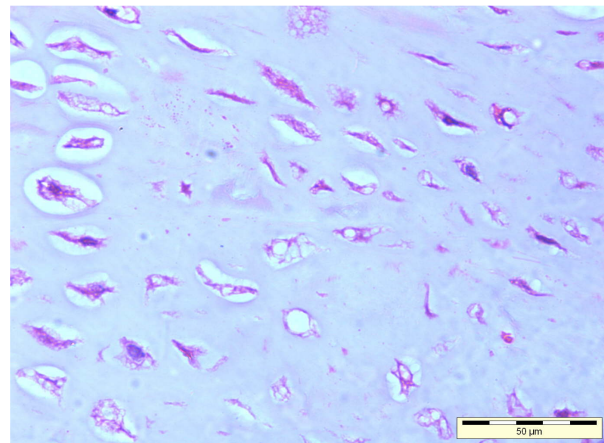


Fig. 8: Group 2 week 8. Close view of hypertrophied chondrocytes surrounding the non hypertrophied ones at the defect centre (x40)

moving from margins to centre the trabeculae become more immature (Fig. 5 and 6). The corticocancellous bone graft was more or less resorped, there was some how a acceptable connection between new bone and old bone. Periosteum had normal configuration and was completely attached to new bone. In control group or group 2 at 8th week, immature bone trabeculae were formed at the margins of the defect and at the centre of defect, hypertrophied chondrocytes was observed indicating that the centre of the defect is still in mid process of endochondrial ossification.

The major pattern detected in control defect was the creeping substitution. The corticocancellous bone graft was seen regularly indicating incomplete resorption of the graft. There was acceptable connection between new bone and old bone but still the demarcation line could be distinguished. On the easier word, defect margins showed immature bone trabeculae and by moving to the centre it

turned into hypertrophied chondrocytes (Fig. 7 and 8). Since the corticocancellous bone autograft had harvested as the scaffold from each animal at the surgery time from iliac wing to assess the healing degree of graft harvesting site, the samples of iliac wing at this region were evaluated histopathologically.

In both control and experiment 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 which could be due to easier harvesting technique that does not require cauterizing.

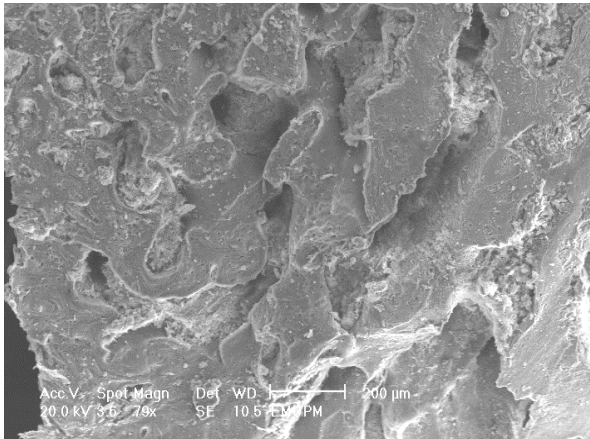


Fig. 9: Group 1 week 8. Mature marginal bone trabeculae covered by osteocytes lacunae

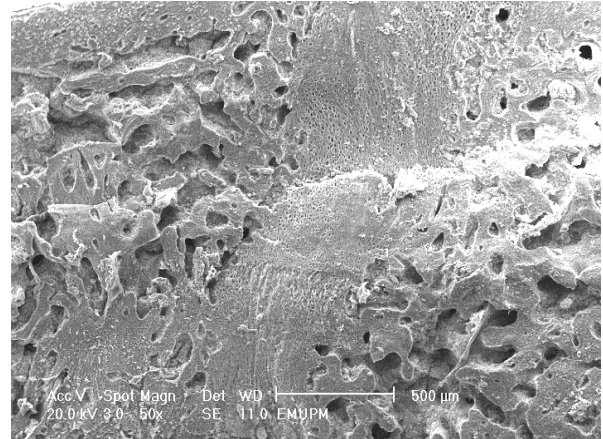


Fig. 11: Group 2 week 8. Hypertrophied chondrocytes at centre and immature bone trabeculae at margins

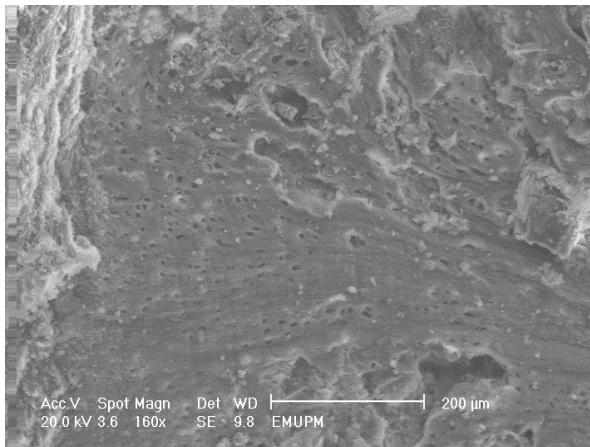


Fig. 10: Group 1 week 8. Immature central bone trabeculae are clear. Hypertrophied chondrocytes are seen (arrow)

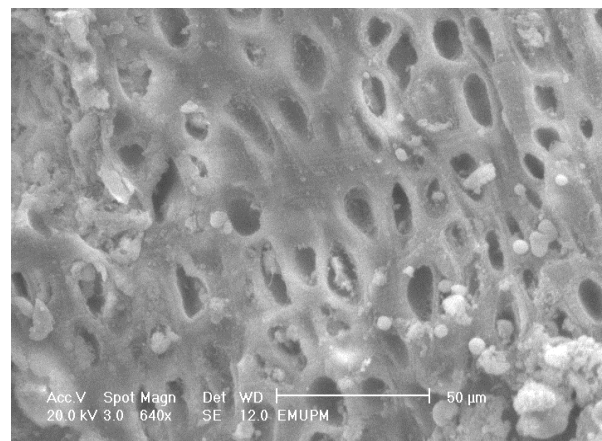


Fig. 12: Group 2 week 8. Hypertrophied chondrocytes from closer view at the centre of defect are obvious

Scanning electron microscope observation: Followed by light microscope, the defect was scanned by scanning electron microscope. In Group 1 at week 8, at margins mature trabeculae was observed that was topped with osteocytes lacunae while middle of the defect was filled with immature trabeculae that were topped with hypertrophied chondrocytes (Fig. 9 and 10). Defect margins in Group 2 showed immature bone trabeculae that were topped with hypertrophied chondrocytes and centre of defect did not manifested bone trabeculae and was just occupied by hypertrophied chondrocytes (Fig. 11 and 12). The result of the study gives the impression to indicate that both the test and control groups 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. Experiment group could enhance the healing processes to a level more advanced than the control group indicating that the experiment group was in level of trabeculae maturation whereas control group was mid level of endochondral ossification that is a level before trabeculae maturity. Also the healing time in these two groups is significantly different.

Radiographic images presented callus as well as new bone formation primarily at the margins of the defect that was gradually pulled toward the center of defect in Group 1 which indicates osteogenesis at both margin and centre of defect during the healing period. However, the newly

formed bone in corticocancellous bone autograft alone was mainly visible at the margins of the defect and the callus formed more slowly than the experiment 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 (Kaveh *et al.*, 2009). By histopathology examination in experiment defect, combination of mature and immature trabeculae (margin and centre, respectively) was observed which approves the radiographic findings. There were large amount of trabeculae penetrated into the centre of 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 were again confirmed. As illustrated, defect in control and test groups had distinct differences with each other indicating complete healing with combination of mature and immature bone trabeculae in Group 1 and incomplete healing or delayed union with combination of immature trabeculae and hypertrophied chondrocytes in Group 2. These data together suggest that osteogenesis, osteoinduction and osteoconduction which are necessary for a good quality and fast healing could simultaneously occur in the experiment group but the osteoinduction, osteoconduction and very little osteogenic (because of the osteogenic 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 cells 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 or growth factors (Rose and Oreffo, 2002). It is believe that the marrow enhanced the bone formation to a level more advanced than bone graft alone due to the presence of osteoprogenitor cells that could be differentiated into osteoblast.

Bone marrow aspirates has two properties; osteogenic and osteoinduction. Bone marrow contains the Osteoprogenitor Cells (OPC) that are important participants in bone formation and fracture healing. Two types of osteoprogenitor cells have been demonstrated; one type that is induced to produce bone (inductive OPC)

and another that is determined to produce bone (determined OPC). The former exists in all connective tissues and is thought to be undifferentiated mesenchymal cells. The latter is found only in marrow and is already differentiated into a bone producing lineage. On the easier word, Osteoprogenitor cells consist of determined osteoprogenitor cells that will become osteoblasts and inducible osteoprogenitor cells that require exposure to an inducing agent. Determined osteoprogenitor cells in their resting state are flattened, relatively undifferentiated mesenchymal cells located on or near all free surfaces of bone including the cambium layer of the periosteum, the endosteum and in the Haversian canals and bone marrow. Determined osteoprogenitor cells are involved in normal bone growth and become activated in bone healing. Inducible osteoprogenitor cells are pluripotent mesenchymal cells which are widely dispersed throughout the body but are more concentrated in muscle and connective tissue (Emami *et al.*, 2002).

An important boundary condition for bone tissue engineering is to obtain the seed cells. Bone marrow has been widely seeded in different scaffolds such as Demineralized Bone Matrix (DBM). Bone marrow contains osteoprogenitor cells and induces bone formation both *in vivo* and *in vitro* (Wang *et al.*, 2003; Kaveh *et al.*, 2009). 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 (Kaveh *et al.*, 2009; Hutmacher *et al.*, 2003; Burg *et al.*, 2000; Zhi and Zu-Bing, 2005).

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 autograft has stimulated its wide use in compact bone augmentation and reconstruction (Kaveh *et al.*, 2009). Altogether, these observations indicate that the combination of autogenous corticocancellous bone graft and bone marrow has the capability of osteogenesis,

osteinduction and osteoconduction with a better osteogenetic effect and quality than corticocancellous bone graft implant alone. In view of the availability of easy and fast method of fresh bone marrow aspiration and also the easy harvesting technique of corticocancellous bone graft from the newly found donor sites with very limited side effects such as distal tibia (Saltrick *et al.*, 1996) as well as using new instrumentation to decrease even that minimum drawback such as acetabular reamer to harvest this graft type (Saltrick *et al.*, 1996) along with high bone regeneration potential, it might be an ideal combination for bone defect repairs. However, In this research it is found 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 (Kaveh *et al.*, 2009).

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 as the origin of osteoblasts has been seeded into the scaffold. It was observed that without *in vitro* differentiation of osteoblasts, bone marrow can also be used as an osteogenic and osteoinductive feature and accelerate the healing procedure. Now there is a challenging question raise here while the bone marrow has the high osteogenecity and osteoinductivity potential 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. Considering the easy and fast BM aspiration technique at the surgery time and also by having shown new sites to harvest corticocancellous bone graft with no donor site morbidity; hence, it might be an ideal grafting combination (fast, easy and cheap which does not require cumbersome MSC isolation, proliferation and osteoblast differentiation) in bone losses and when there is need of excessive osteogenecity potential such as delayed and nonunions.

ACKNOWLEDGEMENT

Different supports of staffs in operation room and radiology laboratory of Faculty of Veterinary Medicine, University of Putra Malaysia are gratefully acknowledged by the researchers.

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