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INTEGRATED EFFICACY OF BIO-OSS® AND DEMINERALIZED BONE MATRIX FOR CRITICAL SIZED ULNA DEFECT HEALING IN A PIGEON (*COLUMBA LIVIA*)

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ABSTRACT

In order to know the mechanism of critical sized defect healing with bone grafts were investigated by radiology and histology in pigeon. Purpose of this study was to estimate the effect of Bio-Oss with DBM for critical sized ulna defect healing in pigeon. Total 12 physically healthy adult pigeons (*Columba livia*) underwent for defect healing using two different bone grafts. Birds were randomly divided into 3-groups of four pigeons in each. Osteotomy procedure was performed using Isoflurane inhalation anesthesia and mixed with O_2. A 1-cm bone piece of left ulna was removed from mid-shaft and filled with Bio-Oss® and demineralized bone matrix (DBM). Birds were sacrificed at their end-point of 3, 6 and 12 weeks for radiographic and histological examinations. Results of this study suggest that combination of Bio-Oss® with DBM for new bone formation in pigeon ulna is effective treatment resulting improved defect healing without any complications. It is concluded that Bio-Oss with DBM may be used for defect healing and osteosynthesis in birds.

Keywords: bio-oss, critical sized ulna defect, demineralized bone matrix, osteosynthesis, pigeon

INTRODUCTION

Bone segment loss due to fracture in birds is a clinical problem, which could be managed by using bone grafts. Bone grafting is the surgical treatment of bone defects used for new bone formation and fracture union (Keating *et al*., 2005; Wong and Rabie, 2010; Liu *et al*., 2013). Bone grafts have ability to restore the skeletal continuity. Scarcity of host bone, alternative such as allografts or Xenografts could be used (Kim *et al*., 2002). Xenograft is prepared from other species and implanted to recipient of different species (Valentine *et al*., 2006). Bio-Oss® (Geistlisch, Wolhusen, Switzerland) is one example of Xenograft contains osteoconductive property and currently used for repair of human as well as for animal defect healing (Zaffe *et al*., 2005). It is a deproteinized natural bone mineral matrix that is prepared from bovine bone (Berglund *et al*., 2000;

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Handschel *et al*., 2009). In recent studies Bio-Oss® has been reported as "gold standard" used for new bone formation (Orsini *et al*., 2005; Schneider *et al*., 2009). Bio-Oss® and autogenous grafts are a suitable combination for new bone formation in rats and humans (Tadjoedin *et al*., 2003; Khoshzaban *et al*., 2010). Demineralized bone matrix (DBM) is an allograft and contains bone morphogenic proteins (Rabie *et al*., 1996; Zipfel *et al*., 2003; Wolfinbarger *et al*., 2008; Pieske *et al*., 2009). DBM is a processed bone graft material used for treatment of non-union cases in human surgery (Jin, 1991). It has osteoinductive and osteoconductive properties and new bone formation occurs through endochondral ossification (Scott and Hightower, 1991; Schwartz *et al*., 2011). DBM has been used first time in birds for new bone formation (Jalila, 2004). The implementation of quick clinical union in fracture is essential to restore the skeletal function of injured birds (Altman *et al*., 1997; Bennett, 1997), but main problem in birds have limited amount of autografts (Bennett, 1997). Therefore, alternative bone grafts such as allografts and xenografts are beneficial for defect healing (MacCoy and Haschеck, 1988; Ozturk *et al*., 2006). To our knowledge, there is no any study reported for mixture of Bio-Oss® with DBM for repair of bone defects in birds. Keeping in view the scope of defect healing with Bio-Oss® and DBM, bone grafts would make things earlier new bone formation in bird fracture cases. This study hypothesized that Bio-Oss® with DBM would provide new bone formation for ulna defect healing in a pigeon model. The purpose of this study was to evaluate the composite capacity of Bio-Oss® with DBM for ulna defect healing in pigeon.

MATERIALS AND METHODS

Birds and bone graft material

All procedures for this experiment were approved by International Animal Care Use Committee of the Faculty of Veterinary Medicine, University Putra Malaysia, (10 R118). Domestic pigeon (*Columba livia*) was purchased from local market of Kuala Lumpur city. All birds were aged between 5-6 months and mean weighed 293.75 g. Two birds were kept in one cage at animal house and acclimatized for two weeks. These pigeons were fed with commercial bird feed (twice a day) and fresh tap water was available *ad libitum*. Two different bone grafts were tested on birds, such as Bio-Oss® (0.25-1mm, Geistlich, Switzerland) was commercially purchased, and demineralized bone matrix (DBM) was prepared from sacrificed pigeons at station. A total of 12 pigeons were randomly separated into three groups' viz. G-1, G-2 and G-3. The duration of bone graft healing was 3, 6 and 12 weeks and four birds in each time. All birds were subjected to receive combination of similar treatment, fracture fixation methods and similar post-operative management.

Surgical procedure

Anesthesia was induced using 4% Isoflurane inhalation anesthesia mixed with oxygen via face mask and maintained at 1.5-2.5% Isoflurane in oxygen 1-1.5 L/min using endotracheal tube (Degernes, 2008). Lactated Ringers solution 10 ml/kg, body weight was administered subcutaneously to all birds. During procedure respiratory rate (breathes/min), heart rate (beats/min) and corneal reflexes were monitored. The feathers from left ulna side were gently plucked

from operative site and prepared using 40% chlorhexidene and 10% povidone solutions. Birds were positioned in sternal recumbency on a heating pad (Conair Corp, East Windsor, NJ, USA) to maintain their body temperature during surgery. A skin incision was made using surgical blade No.13 and ulna bone was exposed. Next, a 1 cm long defect was created in between the ulna of pigeon using ESF pins by inserting pins at the mid-point (Martin and Ritchie, 1994). The pins were drilled 0.5-cm proximally and 0.5-cm distally to the marked mid-shaft of the ulna and 1- cm critical sized defect was created. Post creating defect bone segment was removed and 1-ml sodium chloride 0.9% solution was sprayed on defect site to prevent dehydration. The fracture was stabilized using four external skeleton fixation (ESF) Type-1, size 0.045″ (Imex, Veterinary Inc., TX, USA and fixed perpendicularly one by one to the long axis of the cortex (Redig, 2001). A mini Jacobs chuck was used to insert and fix the pins, two proximally and two distally to the defect (Redig, 2001). After ESF fixation ulna defect was then grafted with combination of Bio-Oss® 50mg granules and 1 cm tubular DBM graft materials (n=12 birds). Wound was closed while using absorbable suture material (Safil-violet 5-0: Polyglycolic acid, Barcelona, Spain) and skin was closed separately. A latex Penrose tube (3/8″ wide and 7 cm long) was inserted over the top of the ESF pins parallel to and above the ulna bone. In last, penrose tubing was filled with a 10 ml mixture of acrylic material (Jorvet™, Jorgensen, Inc., USA). Once the acrylic material had dried completely pins over the column were cut with pin cutter (Jalila *et al.,* 2004).

Post-operative care

All birds were administered with 0.2 mg/kg Torbugesic (2 mg/ml, Fort Dodge Animal Health, USA) as post-operative analgesia. Terramycin antibiotic ointment (Pfizer, Inc. USA) was applied on the incision and the ESF pin site. The wound was covered with a melolin (Smith and Nephew, Ltd.) absorbent pad and a woven gauze sponge to prevent any infection. Operated wing was wrapped with a figure-of-eight bandage and covered with a coban bandage. An oral antibiotic tablet, Noroclav 30 mg (Amoxicillin and Clavulanic Acid, 50 mg), was given to the birds once a day for a week.

Bone graft healing assessment

Post-operative radiographs were obtained to evaluate the defect healing progress at 3, 6 and 12 weeks. All birds were evaluated for bone union, fracture line and callus formation (Table 1). Birds at their endpoint of the experiment i.e., at 3-week (n=4), 6-week (n=4) and at 12-weeks were euthanized with intravenous injection of Pentobarbital 0.3 ml (Lure Cedex, France). Operated left ulna was disarticulated from the bird and healed bone graft along with host bone was collected. The specimens were fixed in buffered 10% formalin solution for 48 hours (Huddleston *et al.,* 2000) and decalcified with 5% formic acid for 3 days with daily changes. Specimens were dehydrated in an automatic tissue processor. All tissue specimens were embedded in paraffin waxes and blocks were mounted into a microtome for sectioning and 5 µm thin sections were made from each. All slides were stained with haematoxylin and eosin for examination of bone graft healing (Savaridas *et al.,* 2012). The slides were viewed under the light microscope and photographed with an Olympus image analyser system

(Olympus®, Tokyo, Japan) and a Virtual slide scanner (Panoramic 3D Histech, Panoramic Viewer, Hungary). The defect site was evaluated for new bone formation, cortex development and bone graft incorporation (Table 2).

Table 1. Radiological scoring criteria used for evaluation of bone graft healing in pigeon ulna

Criterion	Description	Score
Bone formation	No bone formation	0
	Fair	1
	Good	2
	Excellent	3
Fracture line	Visible	0
	Partial visible	1
	Absent	2
Callus formation	No callus	0
	Minimal callus	1
	Extensive callus	2

Source: Ozturk *et al*. (2006).

Table 2. Histological scoring criteria used for evaluation of bone graft healing in pigeon ulna

Criterion	Description	Score
Quality on union	No sign of fibrosis or bone union	0
	Fibrosis tissue union	1
	Immature bone union	2
	Bone union	3
Cortex development	No cortex formed	0
	Formation of new bone along with external border	1
	Incomplete cortex development	2
	Complete formation of cortex	3
Bone graft incorporation	No graft incorporation	0
	Minimal graft incorporation	1
	Moderate graft incorporation	2
	Good graft incorporation	3

Source: Ozturk *et al*. (2006).

Table 3. Radiological results of Bio-Oss and demineralized bone matrix healing in the pigeon ulna. (n= 4 birds) in each endpoint

Parameters	Healing Time	DBM +Bio-Oss mean (SE)
Bone formation	3 weeks	1.00±0.00[a]
	6 weeks	1.00±0.00[a]
	12 weeks	1.25±0.25[a]
Fracture line	3 weeks	1.00±0.00[a]
	6 weeks	1.00±0.00[a]
	12 weeks	1.25±0.25[a]
Callus formation	3 weeks	0.75±0.25[b]
	6 weeks	0.75±0.25[b]
	12 weeks	1.00±0.00[a]

Mean within column with different (a or b) letters are significantly different because of time post bone grafting in birds.

Table 4. Histological results of Bio-Oss and DBM healing in the pigeon ulna.(n= 4 birds) in each endpoint

Parameters	Healing Time	DBM +Bio-Oss (SE)
Quality of union	3 weeks	1.75±0.25[x]
	6 weeks	2.50±0.25[y]
	12 weeks	3.00±0.00[z]
Cortex development	3 weeks	1.25±0.25[x]
	6 weeks	2.00±0.00[y]
	12 weeks	2.50±0.28[y]
Bone graft incorporation	3 weeks	1.50.±0.28[x]
	6 weeks	2.00±0.00[y]
	12 weeks	2.75±0.25[x]

Means within column with different (x, y, z) letters are significantly different because of time post bone grafting in birds.

Statistical analysis

The values were recorded in mean degree with standard error (± SE) for each group (Table 3 and 4). All the tests were performed at 95% confidence level. The statistical analysis was performed using SPSS 21.0 v (IBM®, SPSS® Statistics, Inc. USA).

RESULTS

The results from all operated birds were relatively good during our observation period and birds were recovered quickly after surgery. On gross examination, there was no any evidence of bone graft rejection in any bird (Plate 1).

Radiographic examination

Increased in the radiodensity was observed at 3rd week into the experiment, which have marked bridging of callus over the implanting site without significant bone formation. At 6th week defect was healed with some new bone formation, but still no clinical union occur, however at 12th week, new bone formation and evident callus formation was observed (Table 3) but there was no any significant difference was found in radiological study in any group at any endpoint. Some unclear radiodensity was observed within the defect, because Bio-Oss® contains minerals that can raise the radiodensity (Plate 2).

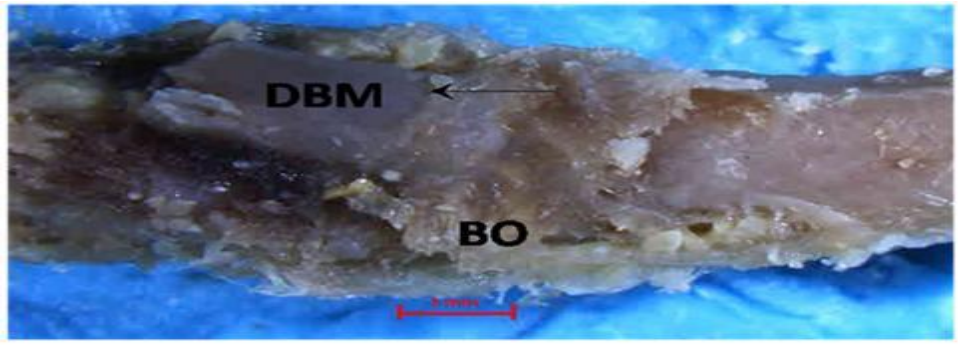

Plate 1. Shows Bio-Oss and DBM grafts healed at 12 weeks in pigeon ulna defect. Above thin arrow indicates DBM (demineralised bone matrix) and blow BO (bio_Oss) show both grafts incorporated and had united with host bone

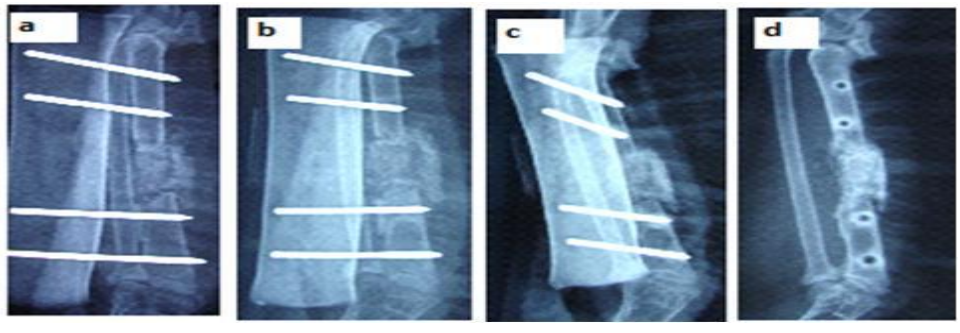

Plate 2. Radiograph of the pigeon ulna defect healed with mixture of Bio-Oss and DBM

(a) Postoperative anterio-postoperative view showing defect filled with borne grafts
(b) After 3-weeks there was slight callus formation
(c) After 6-weeks there was increased mineralization
(d) After 12-weeks bone grafts united with the host borne

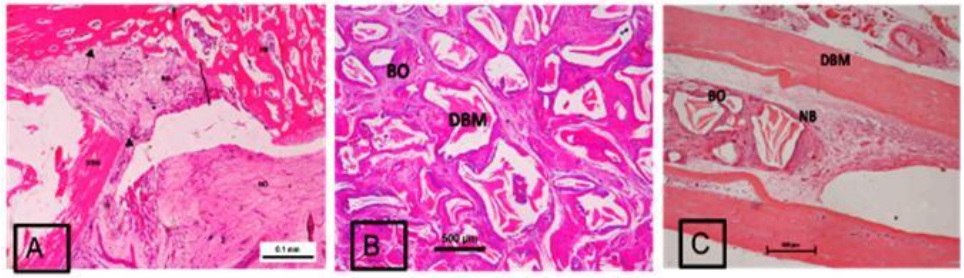

Plate 3. Histological view of defect healed with Bio-Oss® and DBM in pigeon ulna

(A) Immature bone formation seen at 3 weeks of healing (H and E stain, magnification x4).
(B) Trabecular bone formation in defect at 6 weeks (H and E stain magnification x10)
(C) New bone formation and graft incorporation observed at 12 weeks(x4).
B.O = Bio-Oss®, NB = new bone, DBM = demineralized bone matrix.

Histological findings

At 3rd week of the experiment, graft was incorporated into the host tissues and surrounding by fibrous connective tissue. Small amount of new bone formation observed around DBM and Bio-Oss® in defect site (Plate 3a). At week 6 of the defect healing, all birds showed improved bone graft healing. The Bio-Oss® particles were significantly surrounded by newly formed immature bone and connective tissue on the surface. Bio-Oss® formed the trabecular bone which can be identified and it is difficult to identify the bone grafts (Plate 3b). The quality of bone union, cortex development and bone graft incorporation were significantly developed at six weeks (P<0.05), but this development had not led to the complete formation of mature bone or complete cortex in the pigeon model. However, at 12 weeks, all defect showed excellent graft incorporation with each other and attached with the host bone (Table 4). The tubular DBM formed the cortex and extended towards the host cortex. While the Bio-Oss® was replaced in the woven bone with trabecular bone and bone cells. A small amount of bone cells were located around the Bio-Oss® granules, where mineralized bone showing trabecular bone formation at defect site (Plate 3C).

DISCUSSION

In birds, this is a very first time study where Bio-Oss® with DBM were used for new bone formation. By application of such combination our intention was to improve a fracture healing and faster clinical union. It is well known that skeletal gap after fracture will not heal until unless not treated with bone grafts. Demineralized bone matrix (DBM) avian and Bio-Oss bovine bone source were used in this study. A new bone formation was found when Xenografts (Bio-Oss) and allograft (DBM) were implanted for ulna defect healing in a pigeon. This study aimed to evaluate bone formation when critical sized ulna defect was treated with DBM and Bio-Oss. Further this study explain that bone segment loss due to fracture in birds could be replaced with bone grafts. The achievement of clinical union in fracture is necessary in birds (Altman *et al*., 1997; Bennett;

1997). Bone segment loss due to fracture in birds (Bennett, 1997) could be solved by using alternative bone graft substitutes such as DBM and Bio-Oss, which have provided good results for new bone formation as shown in our study. Bone graft healing results over a 3, 6 and 12-weeks post surgery period provide confirmation that granules of Bio-Oss and DBM tubular grafts had provided the structural support for new bone formation in pigeon ulna defect. Previously, experimental fracture healing reported in various animal models such as mouse, rat, rabbit, dog and sheep (An *et al*., 1999). In birds experimental fracture and bone graft healing was conducted on a pigeon model (Jalila *et al*., 2004). In this experiment, we obtained good result from the union of Bio-Oss with DBM as shown in Plate 1. Defect area with new bone formation was evaluated by radiography and histology. Similar assessment methods were used for evaluating experimental fracture healing process in animal model using radiography and histology (Aranson and Shen, 1994; An *et al*., 1999; Ozturk *et al*., 2006). Radiography is frequently used tool to access the fracture healing process. However, radiological examination is non-invasive method to observe fracture healing. Radiographic examination of bone fracture confirms the clinical union and widely used technique. We observed bridging of callus in defect at 3-weeks of healing, while increased in radiodensity due to callus formation was examined at 6-weeks and callus formed around Bio-Oss but bone union was not achieved due to limited time for defect healing. After 12-weeks, bone grafts were united with host bone and completely filled the gap. Callus formation was observed and density of Bio-Oss was advanced on radiographic examination at 12-week of defect healing (Plate 2). Complete mineralization of callus take 4 to 16 weeks after fracture fixation. The results of our study indicate that this combination of bone grafts has induced new bone formation in ulna defect. Orsini *et al*. (2005) also reported that Bio-Oss® provides structural support and has ability for new bone formation. Therefore, Bio-Oss® with autogenous grafts is a suitable combination in rats and humans defect healing (Tadjoedin *et al*., 2003; Khoshzaban *et al*., 2010).

Histological examination of Bio-Oss and DBM at 3-weeks shown, graft incorporation and vascular development with each other which is indication of defect healing. While at 6-weeks bone grafts were surrounded by fibrous connective tissue and immature bone formation was seen. However, similar results were obtained in a rat experiment where Bio-Oss particles were surrounded by fibrovascular connective tissue (Lioubavina *et al*., 2005). After 12-weeks of defect healing woven bone formed in between defects and bone grew inside the defect some cortex developed (Plate 3). Our results suggest that Bio-Oss had formed a visible trabecular bone which can lead to unite the both cortex and fibrous connective tissue formation was due to DBM. Therefore, it was not possible to see radiographic union between bone ends. The findings of this study were supported by Khorsand *et al.* (2012) who found that defect healed with Bio-Oss after 8th week of healing with lamellar and woven bone at defect site. In addition they mentioned that DBM and Bio-Oss groups showed a more regeneration of new bone at 8^{th} week of bone graft healing than 4^{th} weeks of healing. It has been observed that defect healing with bone grafts is time dependent. When birds left for 12-weeks for defect healing, there was better bone formation as compared to 3 and 6 weeks. Unfortunately on histological

examination, Berglundh *et al*. (2000) did not found that Bio-Oss® bone graft incorporation with host for new bone formation for buccal bone defect healing in a dog model. This feature of defect healing with bone grafts is perhaps the most complicated, and the capability to repair a defect model to elucidate how this defect in healing is challenging. It was subjectively observed during study that the combined bone grafts has successfully filled the gap and excellent bone graft incorporation in bird model. DBM contains osteoinductive potency and Bio-Oss® also provides osteoconduction at defect site. To our knowledge, this is first time experiment for assessment of the combined efficacy of Bio-Oss® and DBM for defect healing in birds. The reason of our study was to find out suitable treatment in case of bone loss due to fracture in birds. We observed that the pigeon ulna defects treated with both bone grafts had a constructive effect for new bone formation. Though, it is possible that under clinical condition fracture could be treated with such bone grafts for successful recovery from fracture in birds. A serial radiography and histology confirms the new bone formation.

CONCLUSION

It is concluded that combination of Bio-Oss® with DBM is useful for new bone formation for ulna defect healing in bird model and this could be used for treatment of bone defect healing in wild birds.

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