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# Radiographic Evaluation of Rabbit Femur Implanted Bali Cattle Bone Graft

(EVALUASI RADIOGRAFI FEMUR KELINCI
YANG DIIMPLANTASI BAHAN CANGKOK TULANG SAPI BALI)

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## ABSTRACT

Bone xenograft from cattle bone is commonly used to treat a comminuted fracture case. This study aims to know the process of fractured-femur bone healing in rabbit post-implantation powder bone graft from cortical femur bone of Bali cattle evaluated by radiographs. Ten male local rabbits were used in this study, which were divided into two groups randomly. Group I (KI) as control, the diaphysis of femur bone of the rabbits were drilled with a diameter of 5 mm without implanting the bone graft, while Group II (KII), the diaphysis of femur bone was drilled two holes with a diameter of 5 mm each and with distance 20 mm, substituted with mineralized powder bone graft for the proximal hole (KIIa) and demineralized powder bone graft for the distal hole (KIIb). Fracture healing evaluation was done at week 0 (24 hours), 2, 4, and 6 postoperative by monitoring the growth of callus, fracture line, and union process with radiograph based classification were tabulated statistically, and presented descriptively. The results showed that KI and KII were in the sequel of fracture healing but had not reached remodeling phase perfectly. In conclusion, mineralized and demineralized powder bone graft used in this study was as osteoconductive and can be applied for comminuted fracture.

Keywords: Bali cattle; bone graft; rabbit; radiograph

## ABSTRAK

*Bone xenograft* asal tulang sapi dewasa ini banyak dipakai dalam menangani fraktur kominutif. Penelitian ini bertujuan untuk mengetahui proses kesembuhan fraktur tulang femur kelinci pascaimplantasi bahan cangkok serbuk korteks tulang femur sapi bali yang dievaluasi melalui radiograf. Penelitian ini menggunakan 10 ekor kelinci lokal jantan yang masing-masing dibagi menjadi dua kelompok secara acak. Kelompok I (KI) sebagai kontrol dimana diafisis tulang femur kelinci dibor dengan diameter 5 mm tanpa diimplanbahan cangkok, sedangkan Kelompok II (KII), diafisis tulang femur kelinci dibor dua lubang dengan diameter 5 mm dengan jarak 20 mm, yang diaplikasikan bahan cangkok serbuk mineralisasi pada lubang di bagian proksimal (KIIa) dan bahan cangkok serbk demineralisasi pada lubang di bagian distal (KIIb). Evaluasi kesembuhan fraktur dilakukan pada minggu ke-0 (24 jam), ke-2, ke-4, dan ke-6 pascaoperasi melalui radiograf dengan memonitoring perkembangan pertumbuhan kalus, garis fraktur, dan tahapan penyatuan tulang (union) kemudian diklasifikasikan kemudian diuji secara statistika dan disajikan secara deskriptif. Hasil menunjukkan bahwa KI dan KII mengalami tahapan union namun tidak mencapai fase *remodeling*. Kesimpulannya, bahan cangkok serbuk mineralisasi dan demineralisasi yang digunakan dalam penelitian ini memiliki sifat osteokonduktif dan dapat diaplikasikan pada fraktur kominutif.

Kata-kata kunci: cangkok tulang; kelinci; radiograf; sapi bali

## INTRODUCTION

Fracture is a loss in the continuity of a bone (Syafruddin *et al.*, 2004; Rao, 2010) and causing bone defect (Bigham-Sadegh *et al.*, 2013). Fracture in companion animals found in many small animal clinics yet only few reports it. In the Veterinary Teaching Hospital of Veterinary Medicine Faculty of Udayana University, fracture cases reached 1.8% of 1608 total of patients got into in 2016. Therefore, fracture needs to be treated well. The principle of fracture treatment is to reposition and reconstruct the fractured bone to the normal position and maintained until the healing process ends. One of fracture treatments is using a bone graft, especially in a comminuted fracture case with loss fragments and big defects (Xu *et al.*, 2015). Bone graft was used in the study of Heo *et al.* (2011b) who used swine bone to treat nonunion fracture case with a 40 mm in a diameter, which was a wide defect and healed perfectly in the sixteenth week.

Many used bone graft products are from synthetic but expensive. Therefore, synthetic bone graft needs to be replaced as an alternative. Bovine bone is commonly used for orthopedic treatment in periodontal, maxillofacial, and neurosurgery (Heo *et al.*, 2011a). In this study, we used cortical femur bone of Bali cattle (*Bos sondaicus*) as the basic material of bone xenograft (BX) and evaluated the healing efficacy by implanted it to rabbits as animal model. Healing efficacy was evaluated by adjusting the principle of fracture healing and bone graft as bone substitute. The bone graft usage as bone substitute must be compatible, with the endogenous tissue, non-toxic, non-allergenic, and not inducing inflammation (Chotimah *et al.*, 2013). Fracture healing process was evaluated with radiological examination using x-ray (Xu *et al.*, 2015). Bone of Bali cattle was used in this study since there is no reports of its usage for BX. Moreover, Bali cattle as Indonesian germplasm could be conserved and the bone as a waste from abattoir could be recycled. By implanting powder bone graft from cortical femur bone of Bali cattle to the animal model, we would know the efficacy of the bone graft through its usage and the healing process.

## RESEARCH METHODS

### Preparation of Animal Model

A total of ten local rabbits were used as fracture animal model in this study. Rabbits were male and having the ideal weight for operation, 2500 g±500 g (Pearce *et al.*, 2007; Azam *et al.*, 2016). Animal model in this study had permission as fracture animal model by Ethics Committee, Faculty of Veterinary Medicine Udayana University (No. 293/KE-PH-Lit-1/V.2017). Rabbits were adapted for a week and divided into two groups, Group I (KI) as control (n=5) and Group II (KII) as a treated group (n=5) with KIIa substituted by mineralized bone graft and KIIb substituted by demineralized bone graft.

### Bone Graft Processing

Bone graft processing from a bovine cortical bone in this study was a modification method of Abbas *et al.* (2001). Demineralized bone graft processing was done by clearing the cortical femur bovine bone from muscles and fats with a scalpel and cut into small pieces and smashed with a hammer into powder. Next, the powder was washed in aquadest and NaCl 0.9%. Washed-powder then soaked for 12 hours into chloroform-methanol (1:1 ratio) to defatted and deproteinize. After soaking for 12 hours, powder soaked into HCl 5% for 72 hours at room temperature and HCl was changed every 12 hours with powder weight and solution volume ratio was 50 g : 500 mL. Perfect demineralization was seen to the bone powder consistency became tender and translucent. The powder then washed with aquadest and 70% alcohol. Powder bone graft was preserved in a pot with 70% alcohol in it. Mineralized bone graft processing is the same as demineralized, but not soaked into HCl 5%. The mineralized bone graft was also preserved in a pot with 70% alcohol in it.

### Surgical Procedure

Before getting operated, rabbits were given enough drink without fasting (Flecknell, 2009) and left leg area was shaved. Rabbits received an intramuscular anesthetic of a combination of 5 mg/kg xylazine and 35 mg/kg ketamine (Hau and Hoosier Jr, 2003; Hrapkiewicz *et al.*, 2013;

Enezei *et al.*, 2014; Azam *et al.*, 2016). After anesthetized, animals were placed in a left lateral recumbent position and procedures in a sterile condition.

The skin was incised craniolaterally along the femur bone between trochanter major and patella, the underlying fascia and muscles were retracted to expose the femur diaphysis. Femur diaphysis of each group was drilled with a diameter of 5 mm. During drilling the bone, the bone was washed with sterile saline to prevent heat damage (Azam *et al.*, 2016). Diaphysis femur of KI was drilled one hole without implanting bone graft. Diaphysis was drilled two holes with the distance of each hole was 20 mm, where proximal hole (KIIa) was substituted by mineralized bone graft and distal hole (KIIb) was substituted by demineralized bone graft. Muscles and skin each were sutured with 0000 catgut chromic and 3-0 silk. Postoperation treatment was done by administering antibiotic of 10-30 mg/kg enrofloxacin for 5-7 days and analgesic of 1.5 mg/kg carprofen for seven days orally (Mapara *et al.*, 2012).

**Radiographic Evaluation**

Each rabbit was examined with x-ray for monitoring fracture healing based on the fracture healing classification scoring system according to Whelan *et al.* (2002) from the previous study (Figure 1). Monitoring was done in 0 (24 hours), 2nd, 4th, and 6th week postoperation. The radiograph was taken in left lateral position with 55 kV, 30 mA, and 0.16 seconds with F100 roentgen machine (Amis Medical Co., China) on an 18x24 cm x-ray film (Agfa Radiomat ™, Belgia).

**Data Analysis**

Radiograph results then scored and presented descriptively and statistically analysis by evaluating fracture healing with Kruskal-Wallis test and if there's a significance different ($p<0,05$), the test was continued with Mann-Whitney test.

## RESULTS AND DISCUSSION

Table 2 shows the mean and standard deviation (SD) value of KI and KII fracture

Table 1. Fracture healing classification from radiograph scoring system

| Grade | Callus formation | Radiological assessment | |
|---|---|---|---|
| | | Fracture line | Stage of union |
| 1 | Homogenous bone structure | Obliterated | Achieved |
| 2 | Massive. Bone trabeculae crossing fracture line | Barely discernible | Achieved |
| 3 | Apparent. Bridging of fracture line | Discernible | Uncertain |
| 4 | Trace. No bridging of fracture line | Distinct | Not achieved |
| 5 | No callus formation | Distinct | Not achieved |

Table 2. Value of fracture healing scoring result

| Time (Week) | Score of fracture healing (Mean±SD) | | |
|---|---|---|---|
| | KI | KIIa | KIIb |
| 0 | 5.00±0.00[a] | 5.00±0.00[a] | 5.00±0.00[a] |
| 2 | 3.00±0.00[a] | 3.20±0.447[a] | 2.80±0.447[a] |
| 4 | 3.00±0.00[a] | 2.80±0.447[a] | 2.00±0.00[b] |
| 6 | 3,00±0.00[a] | 2.00±0.00[b] | 2.00±0.00[b] |
| Mean±SD | 3.50±0.889[a] | 3.25±1.164[a] | 2.95±1.276[a] |

Note: KI: control group; KIIa: treated group with mineralized bone graft; and KIIb: treated group with demineralized bone graft. Different superscript shows significance on level $p<0.05$

healing. Scoring of KI shows no significance different of 3.50±0.889 ($p>0,05$). While scoring of KIIa shows significance different on 4th to 6th week of 3.25±1.164 ($p<0,05$) and KIIb shows significance on 2nd to 4th week ($p<0,05$), but no significance on 4th to 6th week of 2.95±1.276 ($p>0,05$).

Radiographic results are presented in Figure 1. In the week 0 (24 hours), KI and KII show no callus formation on the fracture line and the holes were visualized radiolucent. In KIIa, the hole was visualized radiopaque at the center of the hole yet KIIb was not. Both groups in the 2nd week were developing callus formation on the fracture line. In the 4th and 6th week, callus formation was developing massively. In KIIa, the hole was fully covered. Both groups from 0 to 6th week of fracture healing monitoring, the diameter of each hole were visualized narrowed.

Week 0 of KI and KII results show no formation callus on the fracture line. Callus formation was not developed on both groups because 24 hours post operation was still in the inflammation phase of fracture healing process (Hardwood *et al.*, 2010). Inflammation phase of fracture healing process occurs for the first two weeks operation, which marked by bleeding and developing into hematoma and vascularization, which cannot be visualized by radiograph (Pilitsis *et al.*, 2002).

Radiograph of week 0 from both groups shows radiolucent visual. Radiolucent was visualized as the holes when during the surgery the bones were drilled. X-ray emission through the drilled-bones was much more than the area which was not getting drilled. The x-ray then was captured by the film and emulsion on the film was not fixated while processed and resulting radiolucent on the result.


Figure 1. Radiographic results of KI (A) and KII (B), substituted with mineralized bone graft/ KIIa (white arrow) and demineralized bone graft/ KIIb (black arrow). Numbers on figure represent the monitoring week.

Different with KI, KII has two holes and KIIa shows radiopaque on its center. Radiopaque was visualized as the substituted mineralized bone graft to the proximal hole on femur diaphysis. Mineralized bone graft contains more minerals than demineralized bone graft. The mineralized bone graft caused the x-ray emission was absorbed into the bone and emulsion was fixated while processed and resulting radiopaque on the result. However, KIIb shows more radiolucent because the distal gap was substituted by demineralized bone graft which has fewer minerals in it and causing x-ray emission captured by the film and emulsion on the film was not fixated much.

Radiograph evaluation results in the 2nd week show callus formation on both groups. Callus formation in the 2nd week in this study has the same result with the study of Pilitsis *et al.* (2002) when callus formation developed after first two weeks as the repair stage in the bone healing process. Callus formation is a sign of the healing process (Pilitsis *et al.*, 2002; Jahagirdar and Scammell, 2009). In other words, callus formation is one of union process indications where callus scaffolds the fracture line. The visualized callus on the radiograph is known as hard callus (Jahagirdar and Scammell, 2009). Visualization occurs due to hard callus is a differentiation form of mineralized soft callus. The process is called endochondral ossification.

Massive callus formation in the 4th and 6th week on both groups depicts callus development occurred to cover the fracture model. The more callus is developing, the more narrow of the hole diameter is. Callus formation was developing during the evaluation of the fracture model. Callus formation then scaffolds the fracture model with callus and develop into new bone during the healing fracture process.

Radiographic evaluation of femur diaphysis is based on the fractured area which had been scaffold (Corrales *et al.*, 2008). The covered hole by callus is representing secondary or indirect fracture healing (Tosounidis *et al.*¸ 2009). Secondary fracture healing is represented by soft callus formation development into hard callus as a base to scaffold the hole (Tosounidis *et al.*, 2008).

Bone grafts in KII are visualized downward in number until week six. The number was decreased over time due to material absorption and replaced by new bone gradually (Ulum *et al.*, 2014; Azam *et al.*¸ 2016). The new bone replacement was marked by callus formation on the fracture line in both holes.

Based on the results in KII, mineralized and demineralized bone graft from cortical bone of Bali cattle was as osteoconduction rather than osteoinduction or osteogenesis. Osteoconduction can be seen by the holes had been scaffold by the bone graft. Osteoconduction is one of physical properties of bone graft to scaffold the holes (Kalfas, 2001). Likewise, a study of Ramirez-Fernandez *et al.* (2011), which used bovine xenograft (RegenerOss™, Biomet$_3$i, Endobon®, FL, USA), showed that the bone graft their study used was as osteoconduction. One of factors affecting osteoconduction in fracture healing is the granule size of the bone graft (Ramirez-Fernandez *et al.*, 2011). A small-size bone graft eases the bone cells to be scaffold by the bone graft. A study of Bigham *et al.* (2008) which used demineralized bovine bone graft implanted into rabbits also having different significance in fracture healing at 4th week post operation.

An origin from cortical bone of Bali cattle, bone graft in this study is biodegradable and biocompatible. Biodegradable characteristic is proven by bone graft was decreased in number and density, both mineralized and demineralized. The changes of bone graft radio density depict body response in the tissue to the bone graft (Paramitha *et al.*, 2015). Biocompatible is also proven by showing no abnormalities callus growth on radiographs. A material without biocompatible characteristic will result in abnormal tissue growth around the implant (Paramitha *et al.*, 2015).

After evaluated for six weeks, results show both KI and KII were in the sequel of fracture healing, but not perfectly. A perfect fracture healing is achieved after passing the remodeling phase, where bone got the origin shape, structural, and mechanic power (Kalfas, 2001). One of not achieving perfect healing factors is irradiation on the fractured area, especially in the first two and three weeks post operation, because it would inhibit cell proliferation and induce acute vasculitis (Kalfas, 2001).

In the 6th week result of KII, bone grafts are still can be visualized on the radiograph. Cortical bone has fewer osteoblasts and osteocytes, the surface area per weight unit, and its contribution to ingrowth vascularization and remodeling compared with cancellous bone (Kalfas, 2001). In consequence, until week six evaluation, cortical bone grafts used in this study are still many in number due to slower absorption compared with cancellous origin. A cortical bone graft can be perfectly absorbed for years depending on size and local and systemic condition (Roberts and Rosenbaum, 2001).

## CONCLUSION

Both KI and KII were in the sequel of fracture healing and not reached the remodeling phase perfectly. Mineralized and demineralized powder bone graft used in this study was as osteoconductive and can be applied to comminuted fracture case. Mineralized and demineralized powder bone grafts in this study are biodegradable and biocompatible to rabbits.

## SUGGESTION

To another relating research, monitoring fracture healing with x-ray should be evaluated after 4 weeks post operation due to irradiation affects healing.

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