

## Evaluation of equine cortical bone transplantation in a canine fracture model

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**ABSTRACT:** Freeze-dried bovine bone transplantation is commonly used for orthopaedic surgery. Equine bone, which is available in great quantity, can be obtained as easily as bovine bone, and so represents a potential source of bone for transplantation. In the present study freeze-dried equine cortical bones were transplanted into experimentally-induced fibular defects in canines to evaluate xenogenic implantation of equine bone. Cortical bones that had been freed of antigens and defatted with chloroform and methanol were freeze-dried at  $-80^{\circ}\text{C}$  for preservation of bone morphogenetic protein, sterilized with ethylene oxide gas and stored at room temperature. The experimental osteotomy was performed in a 15 mm-long bilateral region of each proximal metaphyseal fibula. The area of defect in eight beagle dogs ( $n = 16$ ) received a transplanted freeze-dried equine cortical implant. The control group consisting of two beagles dogs ( $n = 4$ ) received an autograft of a similar implant. The experiment region was radiographically monitored for bone union and host serum osteocalcin level was determined to assess osteoblast activity every two weeks for 24 weeks. In 14 of the 16 experimental cases, the graft was not associated with new bone formation. Resorption after new bone formation and remodelling with new bone formation each occurred in a single case. The results support the potential of using freeze-dried equine cortical bones as a xenogenic bone graft material in canines.

**Keywords:** equine bone; bone graft; xenogenic bone; bone healing; canine model

In the reconstruction of skeletal defects, it is often necessary to transplant cancellous or cortical bone to restore skeletal integrity and enhance bone healing. The materials used in bone grafting can be divided into autografts, allografts, xenografts, and synthetic materials (Finkemeier, 2002; Parikh, 2002; Cho and Lee, 2006).

In clinical medicine, autologous bone grafting is the best choice for enhancing bone repair and reconstructive bone defects (Burchardt, 1987; Finkemeier, 2002; Parikh, 2002). However, the use of autografts is hampered by the limited supply of bone grafts, the necessity of an additional incision site, donor site morbidity, nerve damage, and injury of the donor site (Younger and Chapman, 1989; Finkemeier, 2002).

Allografts are frequently used in orthopaedic surgery for the treatment of bone nonunion, enhanc-

ing the repair of spine fracture, and reconstruction of bone defects (Sinibaldi, 1989; Berrey et al., 1990; Brady et al., 1999; Haddad and Duncan, 2003). The advantages of allografts include the absence or minimization of donor site morbidity and the unlimited choice of graft shape and size (Finkemeier, 2002; Cho and Lee, 2006). In human medicine, allografts are commonly used, although their expanded use is limited by the supply of bone and the potential for disease transmission (Buck et al., 1989; Hofmann et al., 1995). These limitations have spurred interest in substitute materials.

Recent therapeutic technologies concerning bone substitutes and alternatives to biocompatible scaffolds include growth factor, calcium phosphate, hydroxyapatite, tricalcium phosphate, type I collagen, bioactive glasses, and synthetic polymers. Such materials have been used to fill bone defects in experi-

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mental animal studies and also clinically (Shand and Heggie, 2000; Finkemeier, 2002; Cho and Lee, 2006; Hing et al., 2006; Komaki et al., 2006). The synthetic materials are not osteoinductive and do not induce formation of new bone.

Xenograft bone represents an unlimited supply of available material. Bovine bone transplantation is very commonly used for orthopaedic treatment in periodontal, maxillofacial, and neurosurgeries. Xenograft bone or collagen has been experimentally explored as a bone substitute (Marchesi, 2000; El-Sabban et al., 2007; Stievano et al., 2008). However, the use of bovine bone transplantation is currently limited in human medicine because of the potential for introduction of infection (Boneva et al., 2001; Wenz et al., 2001). In seeking an alternative, equine bone is an attractive potential option. It is available in large quantities and can be obtained as readily as bovine bone. However, the use of equine bone graft in canines has not been studied. This study was undertaken to investigate the use of freeze-dried equine cortical bones as a xenogenic implant in experimentally-induced fibular defects in beagle dogs.

## MATERIAL AND METHODS

### Animals and experimental design

Ten Beagle dogs weighing 9–12 kg (average  $10.1 \pm 1.1$  kg) and 2–3-years-of-age (average  $2.1 \pm 1.7$  years) were used. Physical and radiographic examinations were performed to ensure the absence of orthopaedic diseases. A canine fibula segmental

defect model (Burchardt et al., 1977; Burchardt, 1987) was used to determine the efficacy of the union implants and to compare healing following bone grafting. The basic experimental model consisted of a 15 mm-long, bilateral region proximal metaphyseal fibula segmental defect, which was created in each fibula using an oscillating saw under continuous saline irrigation. Eight dogs received the transplanted freeze-dried equine cortical implant. The remaining two dogs constituted the control, and received an autograft of the transplanted freeze-dried cortical implant. Therefore, the experimental group consisted of 16 cases and the control group consisted of four cases.

### Preparation of freeze-dried cortical bone materials

Cortical bone was obtained from a six-year-old, castrated, male racehorse. The ends of the long bones were cut away with a bone band saw. The midshafts were mechanically scraped clean of soft tissues including cartilage, periosteum, and bone marrow, and washed in cold Ringer's lactate solution. The bone segment (3–5 mm-thick, 20–30 mm-long) was cut to fibula size. The bones were dried and washed with deionized water for 1 h at 4 °C, with periodic stirring. The bones were defatted in a 1 : 1 ratio of chloroform and methanol at 24 °C for seven days, and kept in flowing air for 1 h at room temperature to remove the residual chloroform and methanol by evaporation. The defatted bones were washed by stirring in deionized water for 2 h at 4 °C, and were then frozen at –80 °C. The cortical bone



Figure 1. The antigen-free freeze-dried cortical bones were defatted with chloroform and methanol and were freeze-dried at –80 °C before sterilization with EO gas and storage at room temperature

plate were placed in the drying chamber of a model OPR-FDC-8032 freeze-drier (Operon Engineering, Seoul, Korea) pre-cooled to  $-80^{\circ}\text{C}$ , and a vacuum of  $10^{-2}$  was applied. Complete freeze-drying took about six days, after which the moisture content of the bone was about 5%. The freeze-dried bone was double-wrapped in sterilized packs (one side paper, one side plastic film) and sterilized by exposure to ethylene oxide gas. Sterilized samples were stored at room temperature until use (Figure 1).

### Surgical procedure

Prior to surgery, each patient received 25 mg/kg cephalexin intramuscularly (Methilexin<sup>®</sup>; Union Korea Pharm, Seoul, Korea) for prophylaxis. The experimental animals were premedicated with 0.02 mg/kg subcutaneous atropine sulfate (Atropine Sulfate Daewon<sup>®</sup>; Dae Won Pharm, Seoul, Korea), anaesthesia was induced using 5 mg/kg intravenous propofol (Anepol IN<sup>®</sup>; Ha Na Pharm, Seoul, Korea), and was maintained with 2–3.5% isoflurane and oxygen. The animals were placed in a lateral recumbent position and all surgical procedures were conducted under sterile conditions. A lateral longitudinal incision was made over the fibula and the underlying muscles were retracted, exposing the femur shaft. A 15 mm-long segmental defect was created proximal to the metaphyseal of the fibula using a model BL-F3 electric handpiece saw (Osada Electric, Tokyo, Japan) under continuous saline irrigation (Figure 2). In the experimental group of

eight dogs, each fibular segmental defect ( $n = 16$ ) received a freeze-dried equine cortical implant. In the two control animals, each segmental defect ( $n = 4$ ) was inverted and received an autograft of freeze-dried cortical implant. The muscle attachment was repaired and the skin was closed in layers. Postoperatively a radiography was obtained for all operated ulna to ensure adequate implant placement and alignment. The animals were permitted to freely weight bear immediately following surgery and were allowed to eat.

### Haematology and clinical chemistry analyses

Analyses were obtained on the day of surgery, and every two-weeks for 24 weeks. Clinical and laboratory assessment in all dogs included a physical examination, a complete blood count performed using a Vet Abc automated animal blood counter (ABX Diagnostic, Montpellier, France), a blood smear examination, and basic serum chemistry profile using a Spotchem<sup>TM</sup> SP-4410 automated chemistry analyzer (Daiichi Kagaku, Tokyo, Japan).

### Osteocalcin analysis

Jugular venous blood samples of each dog were collected every two weeks. Samples were individually collected in a 10 ml serum tube. All samples were centrifuged at 3000 rpm at  $4^{\circ}\text{C}$  for 10 min within 1 h

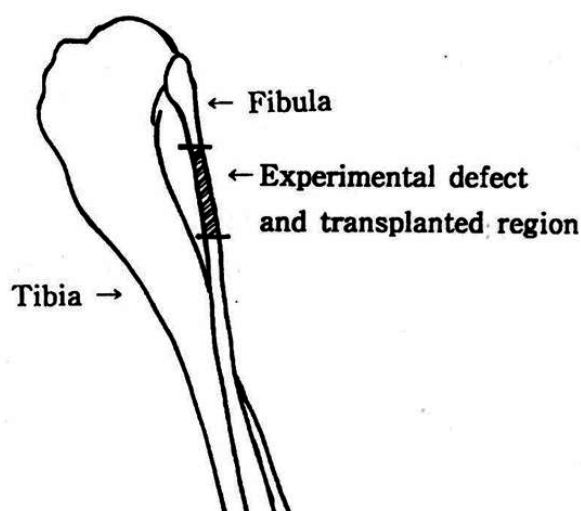


Figure 2. Diagram of the experimental defect and transplanted region of fibula (A). The experimental osteotomy was performed in a bilateral region of the proximal metaphyseal fibula at a length of 15 mm (B)

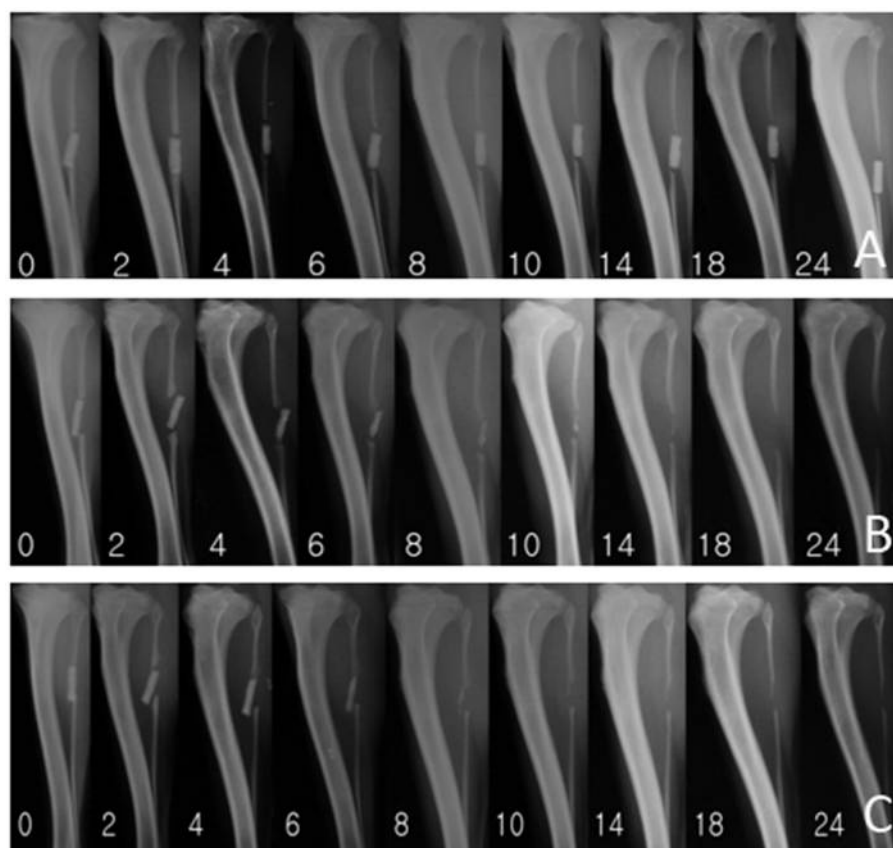


Figure 3. Serial radiographs of hindlimbs with transplanted equine freeze-dried cortical bone in the fibular of dogs. No remarkable changes at the implanted area were observed in 14 cases (A). Transplants with different absorption patterns were found in two cases (B and C)

after sampling. Serum was stored in 3 ml aliquots in a deep freezer at  $-81^{\circ}\text{C}$  until assayed. A Gla-type osteocalcin enzyme-linked immunoassay (EIA) kit (Takara Shuzou, Kyoto, Japan) was used for determination of Gla type osteocalcin concentration according to the manufacturer's protocol. In brief, 100  $\mu\text{l}$  of each test sample was dispensed into wells of a microtitre plate precoated with bovine Gla-osteocalcin standard solution. Following incubation at room temperature for 2 h and washing, detection antibody (mouse mono-

clonal anti-Gla-osteocalcin) conjugated to horseradish peroxidase was applied to each well and incubated for 1 h at room temperature. After washing, 100  $\mu\text{l}$  of substrate solution containing 3,3',5,5'-tetramethylbenzine was added to each well. The plates were incubated for 15 min at room temperature, followed by addition of 100  $\mu\text{l}$  of stop solution. After stopping the reaction with 100  $\mu\text{l}$  of 1N  $\text{H}_2\text{SO}_4$ , the test absorbance at 450 nm was measured with a microplate reader (LabSystem Japan, Tokyo, Japan).

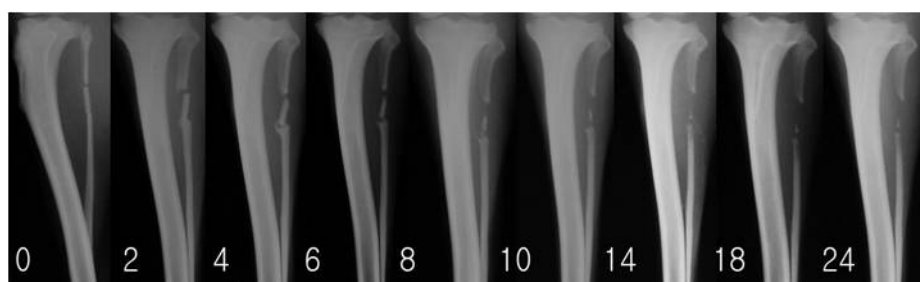


Figure 4. Serial radiographs of implants after transplantation of freeze-dried cortical bone onto an autograft on the fibular of dogs



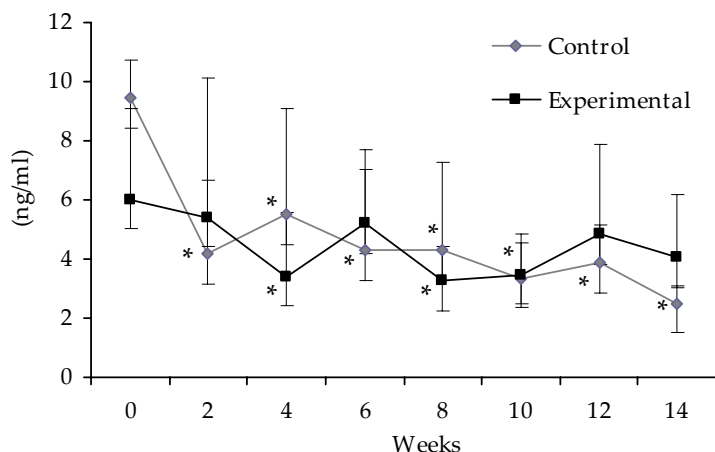


Figure 5. Changes in osteocalcin values in serum after transplantation of freeze-dried cortical equine bone into dogs

\*significant difference in comparison with pre-operative values at weeks 0; ( $P < 0.05$ )

### Plain radiography

The fate of the graft was examined by X-ray every two weeks for 24 weeks. Examined parameters included new bone formation, union of the gap defect, and absorption of bone material.

### Histological evaluation

After 24 weeks, each dog was euthanatized by intravenous administration of sodium pentobarbital (Entobar Inj<sup>®</sup>; Han Lim Pharm, Seoul, Korea). Each

fibula was carefully dissected from the surrounding soft tissue to preserve the bone. Each sample was fixed with 10% formalin, demineralized with 10% nitric acid for 3–5 days, and processed routinely for light microscopy. In brief, the demineralized samples were treated with ascending graded alcohol solutions (70% to absolute alcohol). After treatment with xylene, the samples were embedded in paraffin and sectioned. The 5  $\mu$ m-thick sections were stained with Masson trichrome and observed under a light microscope. Sections were examined for evidence of inflammation and foreign body reaction.

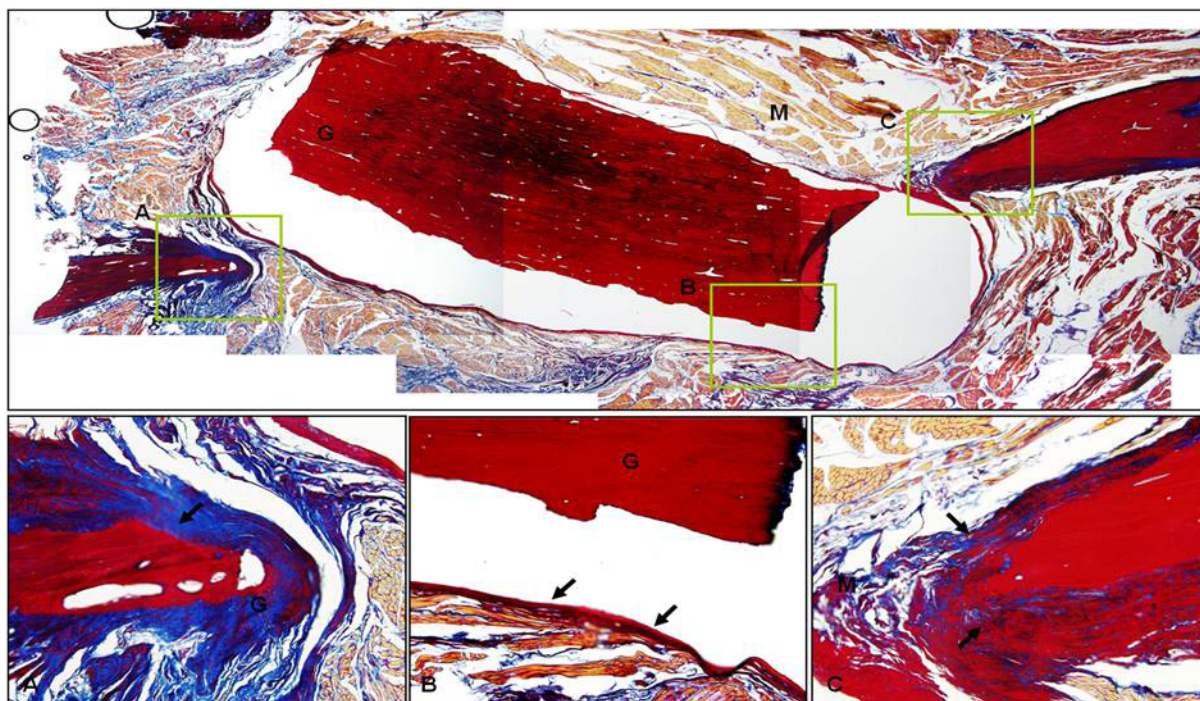


Figure 6. In the xenograft group, the grafted dense cortical bone tissue (G) is isolated by the surrounding of a thin collagenous fibrous capsule (arrows) without bony union. The non-vital grafted bone tissue is not resorbed. No remarkable foreign and inflammatory reactions are noted (trichrome stain, inset original magnification  $\times 100$ )

## Statistical analysis

Results are presented as means  $\pm$  SD. Statistical analysis of data was performed using Student's *t*-test. Values were considered statistically significant when a  $P < 0.05$  or  $< 0.01$  was obtained.

## RESULTS

### Surgical follow-up

All animals recovered fully with no infection or wound inflammation evident. All clinical signs in the study period were within normal ranges.

### Haematology and clinical chemistry analyses

No significant changes were observed. Post-surgical mean values for white blood cell count and liver enzymes were higher in the two control dogs than in the eight experimental dogs at two weeks, but were not statistically significant ( $P > 0.05$ ).

### Plain radiography

In the xenograft group, no remarkable changes at the site of bone transplant were observed in 14 of the 16 cases without any evidence of healing (Figure 3A). In the remaining two cases, the transplant was gradually resorbed (Figures 3B and C). In the autograft group, no evidence of union was found in any of the four cases and the transplants tended to be absorbed more slowly (Figure 4).

### Osteocalcin analysis

During the bone graft period, the osteocalcin serum concentration tended to be lower than the pre-operative level in all groups. In the two control dogs, serum osteocalcin values decreased significantly between weeks 2–14 ( $P < 0.05$ ). In the experimental group, serum osteocalcin values decreased significantly between weeks 4–10 ( $P < 0.05$ ). However, no significant differences were evident between the groups (Figure 5).

## Histological evaluation

No evidence of inflammation and foreign body reaction were observed in either group. In the xenograft group, a longitudinal section in 14 cases demonstrated no evidence of bone bridging, filling of the middle gap with collagenous fibrous or bone union (Figure 6), while in two cases, the longitudinal section demonstrated an irregular margin of bone defect due to resorption and a lack of healing of the bone defect (Figure 7). In the autograft group, the longitudinal section demonstrated no evidence of bone bridging, resorption of cortical bone, or tissue (Figure 8).

## DISCUSSION

The absorption of transplanted bone can take place early following transplantation, can occur later in time, or may be a long lasting absorption without new bone formation (Burchardt et al., 1977; Burchardt, 1987; Choi et al., 1996). In this study, 14 out of 16 equine bone grafts showed no absorption and were without remarkable changes. Implant resorption was found in two cases only.

A rabbit study conducted over three decades ago demonstrated that decalcified cortical bone can induce bone formation intramuscularly in an ectopic site (Urist, 1965). Xenograft experiments conducted in a variety of animal species and graft sites, and with different bank methods have reported enhanced bone formation and bone fracture repair (Anderson et al., 1965; Fujinaga and Koike, 1976; Jensen et al., 2006; Nienhuijs et al., 2006). Mare cortical bone xenografts of low antigenicity have been successfully produced by a cycle of boiling and freezing (Fujinaga and Koike, 1976). Gupta et al. (1982) compared defatted and decalcified xenogenic bone implants in large cortical defects repair and reported a high success rate. Zhao et al. (1998) reported a successful result using bovine bone graft for the reconstruction of anterior base defects in humans. The present results echo those found using bone transplantation allografts. Although less union and formation of callus bridges was observed, the union of distal host bone did occur. No appreciable graft antigenicity was observed either clinically or histologically.

Serum osteocalcin values decreased after transplantation, albeit in a statistically insignificant manner. Increased bone formation in osteocalcin-



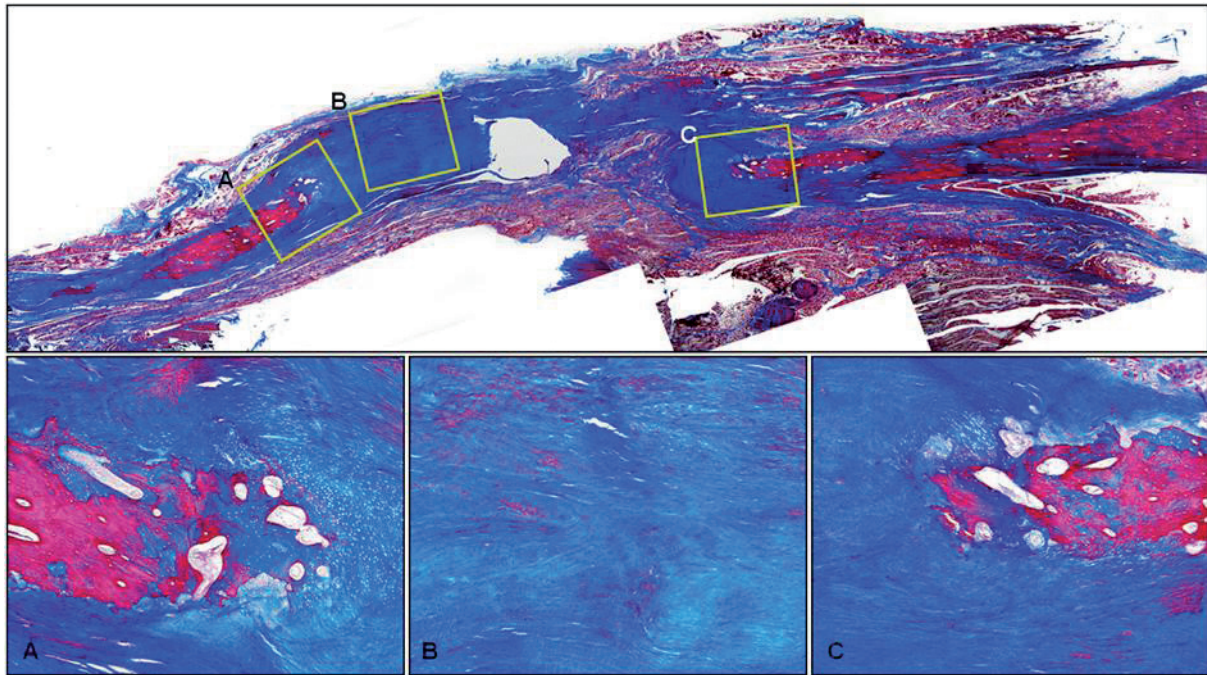


Figure 7. In the xenograft group, the section shows non-union of bones. Both bone margins are resorbed without evidence of reactive bone formation and the gap between the margins are filled with dense collagenous fibrous connective tissue. There is no evidence of inflammatory reactions (trichrome stain, inset original magnification  $\times 100$ )

deficient mice has been reported (Ducy et al., 1996), indicating a suppressive role of osteocacin in bone formation. In contrast, other studies conducted us-

ing canines reported decreased osteocacin values during bone formation (Lammens et al., 1998; Kim et al., 2002).

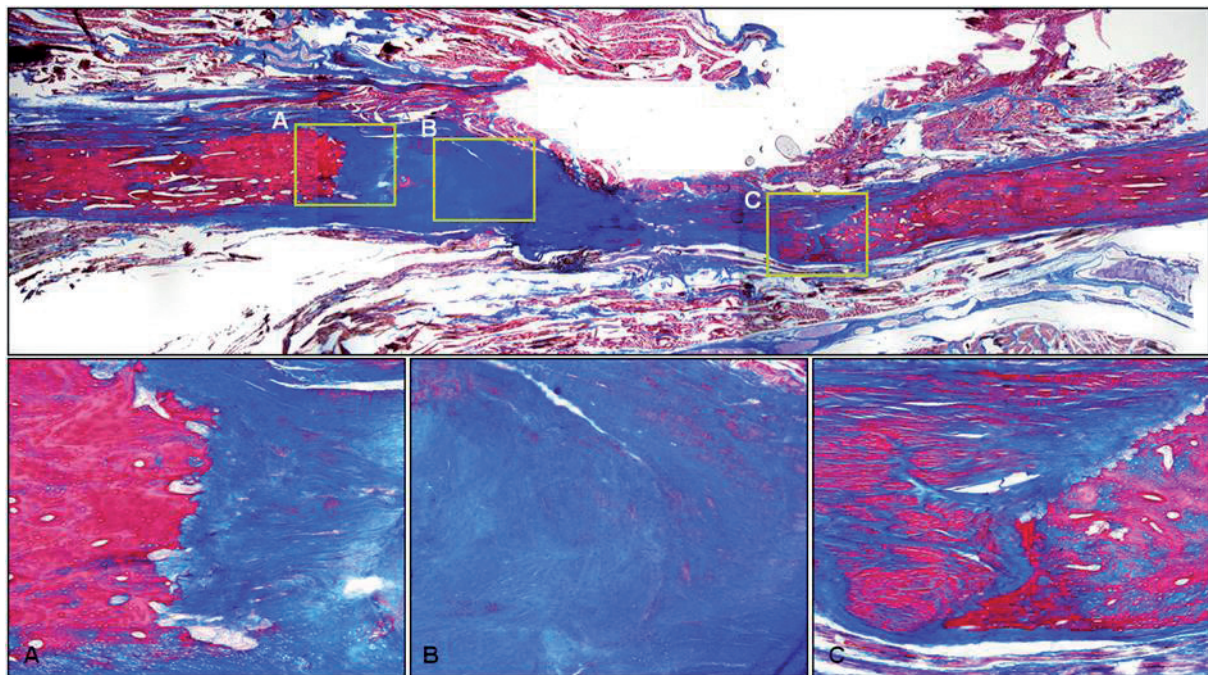


Figure 8. In the autograft group, the gap between the margins of the bone defect is filled with dense collagenous fibrous connective tissue. The margin of the bone defect shows irregular ends due to resorption without evidence of bone healing. No inflammatory reaction is noted (trichrome stain, inset original magnification  $\times 100$ )

Xenografts can be processed to minimize antigenicity. Successful processes include freezing, decalcification, freeze-drying, and deproteinization, with freeze-drying and deep-freezing being most common. Preservation by either freezing or freeze-drying allows extended storage but reduces graft immunogenicity and may alter its mechanical strength. Bone graft preservation aims to maintain the osteoinductive and osteoconductive capacity of material, as well as to reduce its immunogenicity (Friedlaender and Mankin, 1981; Pelker et al., 1984; Burchardt, 1987). The present graft preparations and storage regimens were consistent with these aims.

Lipid extraction by chloroform methanol increases the incorporation of frozen bone grafts (Thoren et al., 1995), perhaps due to a decreased immunologic response. The effect may be also caused by the eradication of major histocompatibility antigens because of cell membrane dissolution (Friedlaender and Mankin, 1981). In this study, inflammation and foreign body response were not observed in the fibula surgical site. However, in the experimental group, repair was characterized by unincorporated, non-union of host bone. The implanted equine cortical bone grafts were not resorbed and no complications ensued.

In conclusion, freeze-dried equine cortical bone has potential as a bone graft material in dogs to alleviate serious bone defects.

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