Comparison of the bone response to xenogenic bone screws and metallic screws in canine femur

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ABSTRACT: The study compared the bone response to xenogenic screws and metallic screws of a similar design in a canine femur. The xenogenic bone screws and metallic bone screws were inserted into the femur diaphyses of dogs. Radiological and histological responses to the xenogenic bone screws were determined in canine bone and micro-computed tomography evaluation of bone mass and bone density parameter was performed 36 weeks after fixation of the xenogenic and metallic bone screws. There was no osteolysis and foreign body reaction associated with either bone screw. The radiological and histological results showed that the xenogenic bone screws formed a bony rim around the screws and facilitated incorporation of host bone. By micro-computed tomography, the bone volume was observed to be significantly ($P < 0.01$) increased at the screw-bone interface surface of xenogenic screws, compared to metallic screws. The bone mineral density of the metallic screw-bone interface was significantly ($P < 0.01$) higher than that of the xenogenic bone screw. The advantages of the clinical application of the xenogenic bone screw are the possibility of its resorption and incorporation. Therefore, additional surgery to remove the screw is not required.

Keywords: xenogenic bone screw; metallic bone screw; micro-CT; canine femur

In clinical medicine, metallic internal fixation is the method of choice for fixation for bone fracture repair and for reconstruction of bone defects. However, metallic internal fixation can result in pain and swelling as the screws loosen, necessitating removal of the metal after fracture healing (Rano et al. 2002; Liptak et al. 2007). Bioabsorbable polymer has been used for fixation in experimental and clinical studies (Eppley 2003; Rasse et al. 2007). The polymer can induce an inflammatory response and osteolytic changes at the implant site (Bostman and Pihlajamaki 2000; Bernard et al 2013). Cortical bone has low antigenic activity and contains different morphogenic proteins including bone morphogenetic protein (BMP), transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Rano et al. 2002; Gut and Sladowski 2007; Liptak et al. 2007). Urist (1965) studied new bone formation after intramuscular implantation of cortical bone

into rabbits and observed new bone formation in a considerable proportion of the implant sites. Other studies have reported allogenic and xenogenic implants for internal fixation devices (Wander et al. 2000; Gut and Sladowski 2007; Liptak et al. 2007; Liptak et al. 2008; Haje et al. 2009). To our knowledge, the bone response to xenogenic bone screws has not been reported in a canine model.

Various techniques that have been used for evaluating the bone response at implant sites include radiography, histology, histomorphometry, mechanical testing, Dual-Energy X-Ray Absorptiometry (DEXA), computed tomography (CT) and micro-CT (Burchardt et al. 1977; Liptak et al. 2007; Okazaki et al. 2008; Wan et al. 2008). Radiography and histological staining can be used to obtain information about the cellular tissue response. However, a disadvantage of radiography and histological evaluations is that they provide only two-dimensional information (2D). High-

resolution three-dimensional (3D) information such as bone mass and bone density parameters can be obtained using micro-CT. On the other hand, metallic implants produce streaking artefacts due to scattering of the micro-CT signal, which prevents an accurate evaluation of bone response after implantation.

The purpose of this study was to investigate the bone response to implants of xenogenic bone screws compared with metallic bone screws in a canine model. Of specific interest were differences in radiography and histological responses to xenogenic bone screws. Micro-CT evaluation of bone mass and bone density parameters was carried out 36 weeks after fixation.

MATERIAL AND METHODS

Implants. Xenogenic bone screws were prepared from the mid-diaphysis of equine tibias. Equine cortical bones that had been cleared of antigens and defatted with chloroform and methanol were freeze-dried at −80 °C, sterilised with ethylene oxide gas and stored at room temperature. Screws were sized by machining to a diameter of 3.5 mm, length of 18 mm and a head diameter of 6 mm (CAMTC, Korea). The metallic bone screws (Apistm Anypia, Korea) used in this study had the same size as the xenogenic bone screws (Figure 1).

Experimental animals and design. Ten beagle dogs (eight intact males, two intact females) aged two to three years (average 2.4 ± 0.3 years) and mean weight 9–13 kg (average 11.2 ± 1.34 kg) were used for this study. Physical and radiographic examinations were performed to ensure the absence of orthopaedic diseases. Xenogenic bone screws

Figure 1. Photograph of a metallic bone screw (left) and xenogenic bone screw (right)

(n = 10) were inserted into the right proximal femur diaphysis of each dog (Group A) and metallic bone screws (n = 10) were inserted into the right distal femur diaphysis of each dog (Group B).

Implantation procedure. The dogs were premedicated with a subcutaneous injection of 0.02 mg/kg of atropin sulphate (Atropine Sulfate Daewon®; Dae Won Pharm, Korea). Anaesthesia was induced by an intravenous injection of 6 mg/kg propofol (Anepol IN®; Ha Na Pharm, Korea), and maintained with isofurane and oxygen. After anaesthesia, the right hind limb was shaved and prepped with betadine and 70% alcohol, and draped with a sterile covering. A longitudinal skin incision was made in the shaft of the bone from the level of the greater trochanter to the level of the patella, and the femur was exposed by a lateral approach between the vastus lateralis and biceps femoris muscle. The periosteum was incised and the mid-shaft of the femur was fully exposed. Subsequently, a hole was made using a 3.5 mm drill bit and tap. The screws were inserted into the femur diaphysis. The implant positions were divided proximal and distal on the femur diaphysis (Figure 2). Following the surgical procedure, the subcutaneous tissues and skin were routinely closed with absorbable and nylon sutures, respectively. Post-operative treatment consisted of intramuscular injection of 10 mg/kg butophanol (Butopan Inj®; Hana Pharm., Korea) every 12 h for three days and an intravenous injection of 25 mg/kg cephalexin (Methilexin Inj®; Union Korea Pharm., Korea) every 12 h for four days.

Plain radiography. The fate of the implant was examined by X-ray every two to four weeks for 36 weeks. Events such as new bone formation, osteolysis, incorporation and screw absorption were evaluated.

Micro-CT. At 36 weeks after surgery, the animals were euthanised by intravenous administration of sodium pentobarbital (Entobar Inj®; Han Lim Pharm, Korea). The femurs were obtained and soft tissues were removed carefully. Screw-bone response of the quantitative evaluation was measured using a Skyscan in-vivo micro-CT instrument (Skyscan n.v., Belgium). The samples were moistened using physiological saline (0.9%) to prevent drying during scanning. Scanning with a resolution of 35 μm was performed at an energy level of 100 KV and intensity of 100 μA. The implants were scanned over 180° in 4° intervals. A 0.5 mm aluminium filter was used to soften and even out the X-ray beams. For reconstruction of 3D images, a cone beam volumetric al-
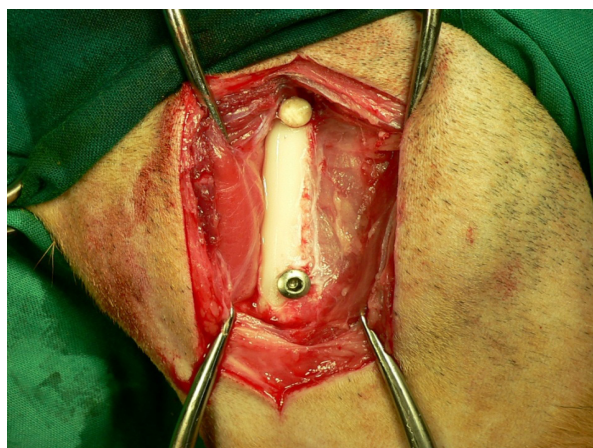
Figure 2. An intraoperative view of femur diaphysis. Xenogenic bone screw of proximal femur diaphysis (group A) and metallic bone screw of distal femur diaphysis (group B)
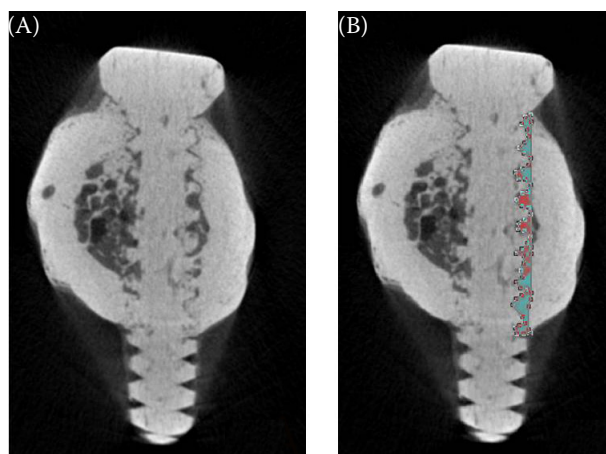

Figure 3. The level of the implanted screw in femur specimens in Micro CT images. (A) Cross-section image, (B) region of interest (coloured) for analysis

gorithm (version 1.8.1.5, CT-analyser Skydcan) was used. Measurements of the 2D images were made on the Region of Interest (ROI). The area of interest was determined and then the bone inside the screw threads was determined. The screw-bone interface surface was calculated, resulting in the ROI (Figure 3). The quantitative bone analysis in this area was measured. Measurements of 3D images were made on the ROI located 30 slices above and below the screw midpoint. The measurements of the metallic bone screws were made upon removal of the implants. Each pair of these parameters in the same specimen was compared for each group. The following parameters were determined using the 3D approach: tissue volume (TV; mm^3), bone volume (BV; mm^3), percent bone volume (BV/TV; %), tissue surface (TS; mm^2), bone surface (BS; mm^2), bone surface/volume ratio (BS/BV; 1/mm), bone surface density (BS/TV; 1/mm), trabecular pattern factor (Tb.Pf; 1/mm), trabecular thickness (Tb.Th; mm), trabecular number (Tb.N; 1/mm), and trabecular separation (Tb.Sp; mm).

Histologic examination. Starting 36 weeks prior to euthanasia, the animals were given 30 mg/kg body weight of tetracycline for 30 weeks and 36 weeks. Each harvested biopsy sample was fixed in 10% buffered formalin and stained with Villanueva bone stain for one week. After dehydration in a graded series of ethanol (70%, 80%, 90%, 95%, and 100%) and infiltration with a mixture of isopropanol and epoxy resin 812, each sample was embedded in epoxy resin 812 and polymerised in a 60 °C oven for seven days. The polymerised block was cut in a bucco-lingual or mesio-distal direction using an

Accutom-50 hard tissue cutting machine (Struers, Germany) under constant cooling. The undecalcified sections had an initial thickness of around 150 μm and were successively ground to a thickness of approximately 50 μm using a Rotopol-35 grinding machine (Struers, Germany). The sections were examined by fluorescence microscopy (Olympus, Japan).

Statistical analysis. An unpaired Student's *t*-test was used to compare the data of the experimental group (Group A) and control group (Group B). A *P*-value < 0.01 computed using SAS version 9.1 software (SAS, USA) was considered significant. The data were expressed as mean ± standard deviation (SD).

RESULTS

Surgical follow-up

All dogs tolerated surgery and fully recovered without infection and additional surgical procedures. Clinical signs in all dogs in this study were within the normal range. All dogs were able to fully bear weight on the operated limb.

Plain radiography

Group A. At six weeks after surgery, a bony rim around the screws and new bone formation was seen in four cases. Eight weeks after surgery, screws were absorbed partially in two cases. The exterior
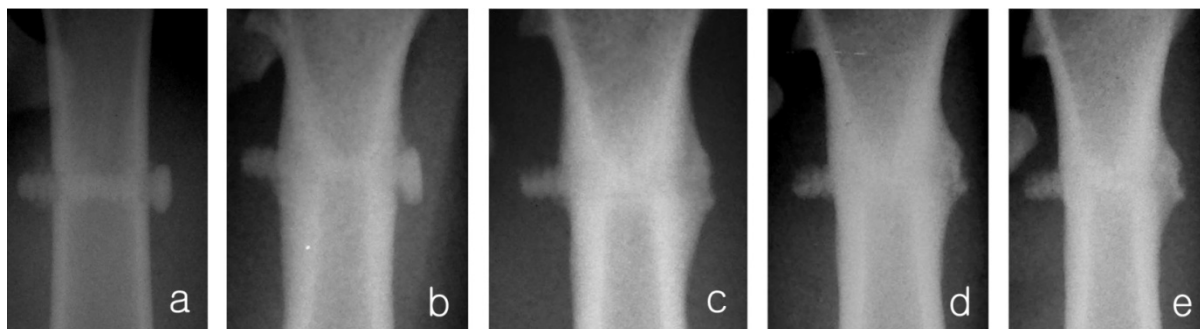
Figure 4. Serial mediolateral radiographs of femur inserted with xenogenic bone screw (group A). Note the bony rim around the screw. (a), (b), (c), (d), and (e) depict the view at 0, 4, 8, 26, and 36 weeks after surgery, respectively
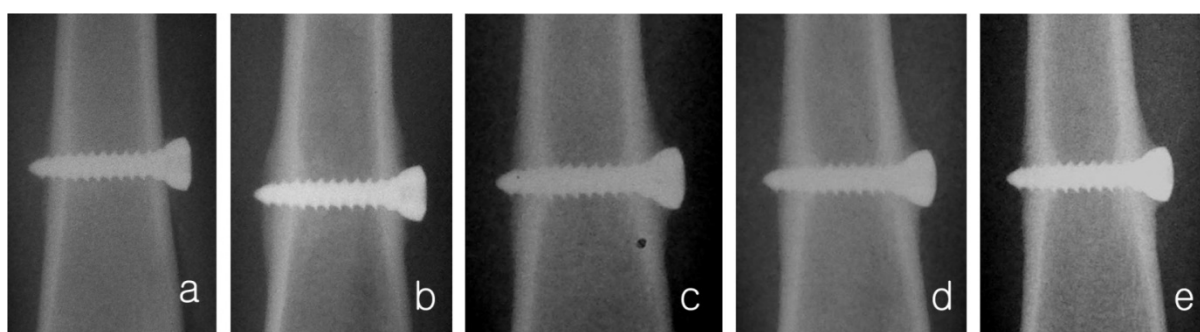

Figure 5. Serial mediolateral radiographs of femur inserted with metallic bone screws (group B). No change in the screw was seen at 36 weeks. No signs of osteolysis and foreign body reaction were observed. (a), (b), (c), (d), (e) depict the view at 0, 4, 8, 26, and 36 weeks after surgery, respectively

of the screw was absorbed completely by 26 weeks in one case. At 36 weeks, a bony rim around the screws and new bone formation was observed in six cases. Moreover, the screw was absorbed partially in one case, completely in another and displayed no change in two cases. Osteolysis and foreign body reaction were never observed (Figure 4).

Group B. Slight periosteal reaction was observed in eight cases at four weeks, but little change around the screws was evident at 36 weeks (Figure 5).

Micro-CT

Group A. After 36 weeks, xenogenic screws were replaced by bone in the screw hole of all specimens. A bony rim around the screws and incorporation was observed in six cases. The screw was incorporated partially in three cases and absorbed completely in one case (Figure 6).

Group B. The appearance of the metallic screws was unchanged in all specimens. No signs of in-
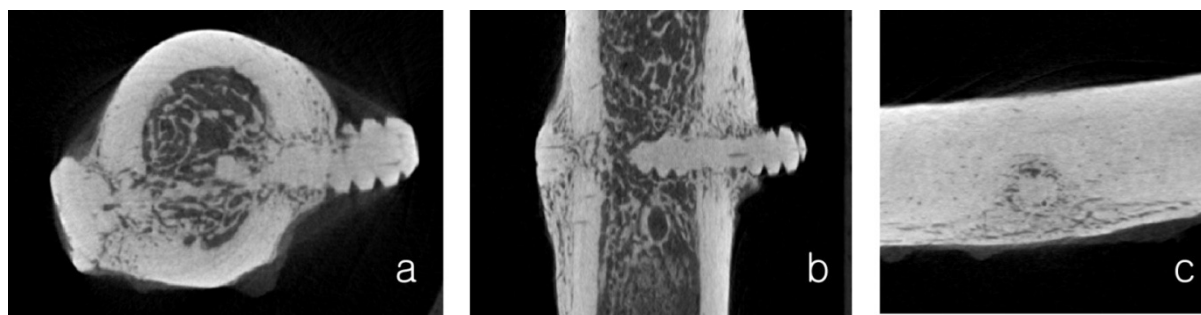

Figure 6. Micro-CT images of a xenogenic screw at the implant site in group A after fixation. A bony rim around the screws and incorporation were observed at 36 weeks. No signs of osteolysis and foreign body reaction were observed. Cross section (a), longitudinal section (b), saggital section (c)

Table 1. Comparative 3D micro-CT analysis of screw-bone interface surface at 36 weeks

Parameter	Group A (n = 10)	Group B (n = 10)
Tissue volume (TV, mm^3)	4.12 ± 0.77*	11.25 ± 2.44
Bone volume (BV, mm^3)	2.19 ± 0.37	2.42 ± 0.66
Percent bone volume (BV/TV, %)	54.29 ± 12.25*	22.42 ± 7.58
Tissue surface (TS, mm^2)	39.35 ± 4.2	54.25 ± 5.2
Bone surface (BS, mm^2)	32.78 ± 5.45*	27.81 ± 6.72
Bone surface/volume ratio (BS/BV, 1/mm)	15.19 ± 2.1*	11.6 ± 1.35
Bone surface density (BS/TV, 1/mm)	8.17 ± 1.89*	2.56 ± 0.81
Trabecular thickness (Tb.Th, mm)	0.34 ± 0.05	0.38 ± 0.05
Trabecular separation (Tb.Sp, mm)	0.34 ± 0.07*	0.6 ± 0.14
Trabecular number (Tb.N, 1/mm)	1.61 ± 0.46*	0.58 ± 0.18

Data are mean ± SD; *P < 0.01, compared with Group B

flammation and foreign body reaction were detected around the xenogenic and metallic screws.

The results of quantitative morphometric analysis of micro-CT are presented in Table 1. In comparative 3D micro-CT analysis, Group A displayed significant changes compared to Group B. The total volume of new bone was 54.29 ± 12.25% in Group A, which was significantly (P < 0.01) higher than in Group B (22.42 ± 7.58%); its volume was 2.19 ± 0.37 mm^3. In Group A, BS (32.78 ± 5.45 mm^2), BS/BV (15.19 ± 2.1 l/mm), BS/TV (8.17 ±1.89 1/mm), and Tb.N (1.61 ± 0.46 1/mm) were significantly greater than values in Group B (BS, 27.81 ± 6.72 mm^2; BS/BV, 11.6 ± 1.35 1/mm; BS/TV, 2.56 ± 0.81 1/mm; Tb.N 0.58 ± 0.18 1/mm) (all P < 0.01). TV and Tb.Sp in Group A (4.12 ± 0.77 mm^3 and 1.61 ± 0.46 mm, respectively) were significantly lower than in Group B (11.25 ± 2.44 mm^3 and 0.6 ± 0.14 mm, respectively) (all P < 0.01). No significant differences (all P > 0.05) were evident in BV, TS, and Tb.Th between Group A (BV, 2.19 ± 0.37 mm^3; TS, 39.35 ± 4.2 mm^2; Tb.Th, 0.34 ± 0.05 mm.) and Group B (BV, 2.42 ± 0.66 mm^3; TS, 54.25 ± 5.2 mm^2; Tb.Th, 0.38 ± 0.05 mm).

Histological examination

Group A. At 36 weeks after implantation of the xenogenic bone screws, new bone formation and incorporation in host bone were observed. Newly formed bone tissues and numerous osteon formations were seen in the xenogenic bone screw and host bone. Xenogenic bone screws displayed yellowish-green fluorescence (Figure 7).
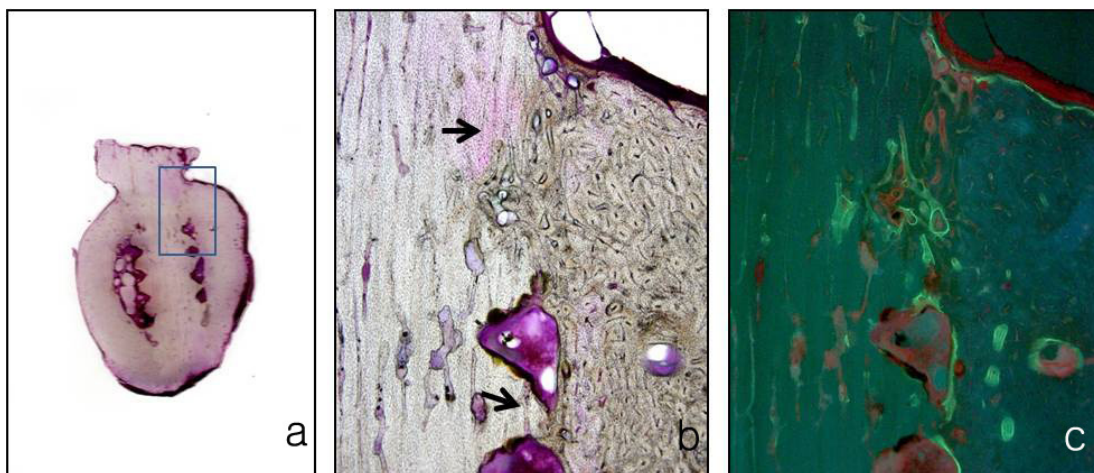

Figure 7. Representative histological views after fixation with screw of the implant site in group A. Villanueva stain original magnification × 1 (a), Villanueva stain original magnification × 40 (b), fluorescence micrograph original magnification × 40 (c). Incorporation of xenogenic bone screw into host bone (black arrows)
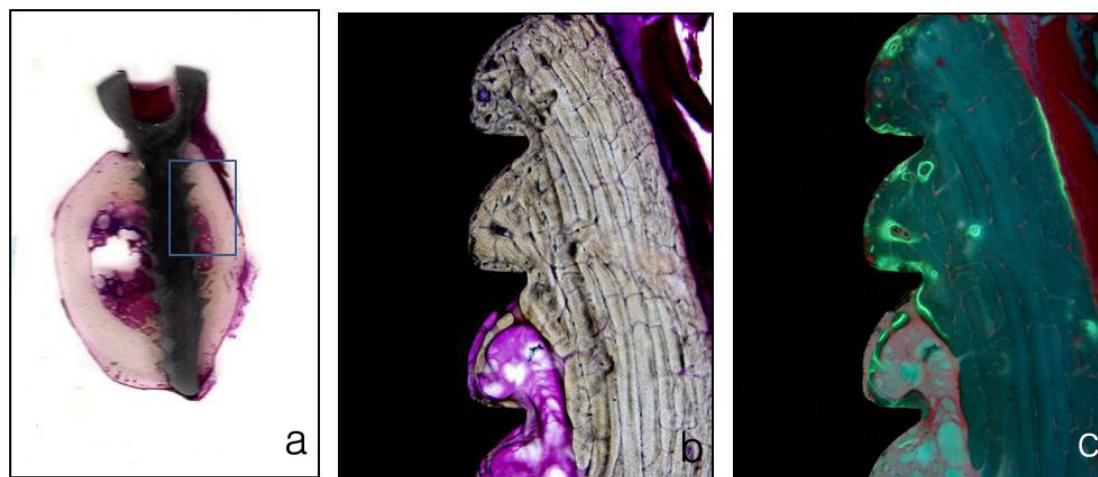
Figure 8. Representative histological views after fixation with screw at the implant site in group B. Villanueva stain original magnification × 1 (a), Villanueva stain original magnification × 40 (b), fluorescence micrograph original magnification × 40 (c)

Group B. After 36 weeks implantation of metallic bone screws, new bone formation in host bone was observed. Metallic bone plate of lamellar bone had formed at the interface between host bone and implants. The host bone displayed yellowish-green fluorescence (Figure 8).

DISCUSSION

In this study, we observed a bony rim around xenogenic bone screws as well as incorporation of host bone. There was no osteolysis and foreign body reaction with either the xenogenic or metallic bone screws.

Incorporation of a cortical bone graft is a lengthy and complex biological process underpinned by interactions between the grafts and host bone. Factors that are influential for incorporation are revascularisation, new bone formation and host-graft union (Stevenson et al. 1996). Numerous studies have attempted to successfully incorporate allografts, xenografts and autografts in animals. A study of freeze-dried fibular allografts in dogs reported the grafts to be biologically and mechanically inferior to similar autografts (Burchardt et al. 1978). Another study demonstrated vascularisation, new bone formation and incorporation when autografts, allografts and xenografts were used to treat tibia defects in rabbits (Ozyurek et al. 2008).

The present radiological and histological results show that new bone formation occurred, with delayed absorption in 90% of the cases and complete absorption in 10% of the cases. The optimistic aim of a bone graft is to minimise the immunological reactions that take place in the host, to promote new bone formation and to instil or improve mechanical strength. Here, the cortical bone was processed to ensure low antigenicity in a process involving freezing, decalcification, freeze-drying and deproteinisation. This regimen is common. Freezing or freeze-drying are both preservation techniques that allow extended storage and reduce the immunogenicity of the graft; in addition, they may also alter its mechanical strength. Bone grafts are typically processed and preserved with the intent of maintaining the osteoinductive and osteoconductive capacities of the material, and of reducing immunogenicity (Friedlaender and Mankin 1981; Pelker et al. 1984; Hubble 2001).

Lipid extracted with a solution of chloroform and methanol in cortical bone is typically used to suppress immune response for bone implants that are stored in bone banks. Lipid extraction by chloroform methanol has been reported to increase the incorporation of frozen bone grafts. This effect may be caused by a decreased immunological response or by eradication of major histocompatibility antigens because of cell membrane dissolution (Friedlaender and Mankin 1981; Thoren et al. 1995). It has been reported that 25% of bone delipidised by acetone can be resorbed and can replace new bone at four weeks (Urist and Mikulski 1995). Transplantation of freeze-dried chloroform-methanol treated autogenic and xenogenic bone to tibia can result in a resorption rate exceeding

50% and formation of new bone at 20 weeks (Choi et al. 1996; Choi et al. 1998). The present study shows that resorption and incorporation of cortical bone screws is promoted by suppression of immune response, delay of absorption due to bone delipidisation and maintenance of BMP by freeze-drying.

Previous studies have attempted to biomechanically characterise cortical bone implants and to compare bio-absorbable and stainless steel implants. In one such study, allogeneic cortical bone pins of the canine tibia were compared to stainless steel pins and polydioxanone pins using impact testing and four-point bending. Bending properties of cortical bone pins were significantly better than polydioxanone pins, but were significantly worse than stainless steel pins (Liptak et al. 2008). Haje et al. (2009) reported the possibility of manufacturing screws made of bovine cortical bone delipidised by ethyl ether. Chemically processed screws held higher ultimate loads under bending and torsion than untreated bone. Moreover, threading significantly decreased the torsion strength of the screws. However, biomechanical testing of cortical bone differs from clinical conditions because it does not address on-going biological processes associated with vascularisation, new bone formation and incorporation.

Micro-CT is a non-destructive technique for visualising bone implant response. 3D images of a sample that can be computationally rotated and viewed from any angle can be produced. An additional advantage is that image processing algorithms that are free from model assumptions used in 2D histomorphometry have been developed to segment and quantitatively characterise bone responses in three dimensions (Shefelbine et al. 2005; Morgan et al. 2009). In our experiments, the bone volume increased the screw-bone interface surface of the xenogenic screws (Group A) as compared to the metallic screws (Group B). In 3D imaging, the total volume of new bone in Group A was 54.29% while the new bone volume in Group B was 22.42%. Thus, our results support the contention that the graft provides for active bone formation and induces cells of the surrounding soft tissue to form bone and to serve as a substrate for new bone formation.

In conclusion, the present study shows that the deceased BMD of xenogenic screws is likely caused by new bone formation and the delayed absorption of screw-bone interface. Conversely, the metallic screw-bone interface facilitated complete bone formation during bone remodelling. Clinically, xenogenic bone screws offer an advantage of possible resorption and incorporation, which would preclude the need for a second surgery to remove the screw. Xenogenic bone screws are reliable tools for internal fixation in fractures because they promote healing of fractures and maintain the fixation of the bone for more than 30 weeks.

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