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Bovine bone implant with bovine bone morphogenetic protein in healing a canine ulnar defect

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Abstract Xenograft is considered an alternative material for bone transplantation, but its bone healing capacity is inferior compared to that of autografts and allografts. Here, we tested whether bone morphogenetic protein (BMP) addition enhances the suitability of demineralized xenogeneic bovine bone for bone grafting in dogs, and whether xenogeneic bone is a suitable carrier material for BMPs. The capacity of demineralized bovine bone implants, with and without native partially purified bovine BMP, to heal a 2-cm ulnar defect was determined in six dogs over a follow-up time of 20 weeks. No instances of bone union were seen, but there was slightly more bone formation in the xenografts with BMP, though the difference was not statistically significant. The ulnas treated with an implant with BMP were also mechanically stronger, but the difference was not significant. Computed tomography scans showed no differences in the implant area in bone density, bone mineral content, or bone cross-sectional area. It is concluded that native, partially purified BMP does not sufficiently improve the suitability of bovine demineralized xenografts as a bone substitute material for dog. Demineralized xenogeneic bone does not seem to be a feasible carrier material for BMP.

Résumé Les xénogreffes sont considérées comme des matériaux alternatifs aux transplants osseux, mais leur pouvoir réparateur pour l'os est inférieur à celui des autogreffes et des allogreffes. Nous avons cherché à déterminer si l'ajout de la protéine de morphogenèse osseuse (PMO) améliore la performance de l'os bovin xénogénique déminéralisé en tant que greffes osseuses chez le chien, et si l'os xénogénique constitue un véhicule efficace pour la PMO. Nous avons déterminé le pouvoir réparateur des implants osseux bovins déminéralisés, avec ou sans supplément de PMO native bovine partiellement purifiée, dans la guérison d'une résection ulnaire de 2 cm chez six chiens, sur une période de suivie post-opératoire de 20 semaines. L'union osseuse n'a été observée en aucun cas. Cependant, une formation osseuse plus abondante était observée dans les xénogreffes avec un supplément de PMO, mais la différence n'était pas statistiquement significative. Les os ulnaires traités avec une greffe à la PMO étaient aussi mécaniquement plus résistants, mais la différence n'était pas statistiquement significative. L'analyse tomographique n'a révélé aucune différence de densité osseuse, ni de composition minérale, ni de surface de section osseuse, dans la région de l'implant. Nous concluons que la PMO native partiellement purifiée n'améliore pas suffisamment l'aptitude des xénogreffes bovines déminéralisées à constituer un matériel de remplacement osseux chez le chien. L'os déminéralisé xénogénique ne semble pas être un véhicule efficace pour la PMO.

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Introduction

Autologous bone harvested from the iliac crest is a commonly used grafting material. There are, however, problems due to morbidity at the harvesting site and the availability is limited. The same is also true for allografts, where a growing awareness of disease transmission has led to a search for alternative materials.

Xenogeneic bone is available in unlimited supply. However, it has to be processed to render it safe for transplantation to a human host [1]. Xenogeneic bone, usually of bovine origin and processed in various ways, has been used in many animal studies [3, 6, 7, 13, 14, 19]. The results have been acceptable but generally inferior to autogeneic bone. Most authors consider processed xenogeneic bone a possible bone grafting material, but

the need for more systematic studies is obvious, especially in view of the effects of added growth factors.

Bone morphogenetic proteins (BMP) constitute a large family of proteins which are osteoinductive and able to produce bone at an ectopic site [18]. We have previously found that BMP enhances the capacity of a coral implant to heal a segmental ulnar defect by increasing bone formation [16]. Therefore, it is possible that adjunction of BMP to a xenograft might improve its usability as a bone substitute.

The aim of this study was to test in the canine ulnar defect model, which is considered suitable for bone substitute and transplantation studies [4, 8, 12, 15, 16], whether BMP addition enhances the suitability of demineralized xenogeneic bovine bone for bone grafting and whether xenogeneic bone is a suitable carrier for BMPs.

Materials and methods

Six laboratory-bred beagle dogs were used, both male and female, aged 1 year, weighing 9.0–12.0 kg. All the experimental manipulations were approved by the Committee on Animal Experimentation of Kuopio University.

The xenogeneic implant, 9 mm in diameter and 20 mm in length, was manufactured from demineralized bovine cancellous bone. Demineralization was performed in 0.6 N HCl (+4°C for 3 days). After that, the implant was placed in 10% hydrogen peroxide at room temperature for 24 h. The BMP was extracted from bovine diaphyseal bones as described earlier [5]. This partially purified BMP, including a combination of several growth factors, was used at a dose of 30 mg per implant, and BMP was adsorbed to the bovine bone implant. The activity of the extracted BMP was tested prior to the implantation in a rat thigh muscle poach model. The implants were sterilized with ethylene oxide.

The operations were made under general anesthesia using pentobarbital (Mebunat, Orion-Farmos, Helsinki, Finland) in a dose of 15 mg/kg intravenously. Xylazine (Rompun Vet, Bayer, Germany) at 1 mg/kg was used as preoperative premedication. Both forelegs were prepared and draped in a sterile fashion. A rubber band was used as a tourniquet above the elbow joint. A lateral incision was made and the ulna exposed. Using an oscillating saw, an osteotomy including the periosteum was made in mid-ulna and a 2 cm defect was inflicted.

Plate fixation was performed with a 10-hole stainless steel miniplate and screws (Stratec Medical, Oberdorf, Switzerland), applying three screws proximally and distally of the defect; four middle holes were left empty The defect of the left ulna was filled with a pure xenograft implant and that of the right ulna with a xenograft implant with BMP.

Pain medication after the operation consisted of buprenorfin (Temgesic, Reckitt&Colman, Hull, UK) at 0.01 mg/kg intramuscularly.

The dogs tolerated the operation well, and weight bearing began on the first postoperative day. The dog chow was Serti (Suomen Nestle, Helsinki, Finland). The dogs were kept in separate cages for 1–2 days after the operation and then in large outdoor/indoor runs with shelter for the duration of the study.

The dogs were killed after 20 weeks with an overdose of pentobarbital (Mebunat) 60 mg/kg intravenously. The ulnas were dissected out and the soft tissue removed. The bones were wrapped in saline and frozen at –20°C until analysis.

The position of the implant and the fixation material were checked postoperatively with roentgenograms. Bone healing was evaluated with further X-rays by taking both antero-posterior and lateral views after 3, 6, 9, 12, 16, and 20 weeks. Two investigators estimated bone union and bone formation independently. The cases of disagreement were reviewed together. The interpretation was blinded between the two groups.

In the evaluation of bone union, we used the scoring system proposed by Johnson et al. [9], in which proximal union was graded as 0–3 and distal union as 0–3 [9]. Thus, the highest score possible for bone union was 6. Bone formation was also scored, the maximum score being 4 [9].

The ulnas were thawed at room temperature for densitometry and mechanical testing. During the testing, the bones were kept moistened to avoid the potential effect of drying on the mechanical properties [17].

The bones were scanned using a peripheral quantitative computed tomographic (pQCT) system Stratec XCT 960A with the software version 5.20 (Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). A voxel size of 0.295×0.295×1.25 mm^3 was used. We scanned five slices of each sample: one slice in the middle of the implant, two slices at the distal and proximal bone-implant border areas, and two slices outside the implant from the distal and proximal ulna near the bone-implant border, the slice positions being defined from the axial scout view of the pQCT system. Total and cortical bone mineral density (BMD), total and cortical bone mineral content (BMC), and total and cortical bone cross-sectional area (CSA) were recorded using an attenuation threshold of 0.7 cm^{-1} to define cortical bone.

The mechanical strength of the bones was evaluated by torsional testing. The bone ends were embedded into moulds with two-component fibreglass resin, using a torsional shaft of 8 cm. After hardening of the resin, the bones were placed in the torque machine and torsionally loaded at a constant angular speed of 6.5°/s until failure [10]. Maximal torque capacity (MTC) was recorded.

After torsional testing, the bones were reconstructed and a 4–5 cm long section, including the implant site, was taken for histological analysis. After fixing in 10% neutral formaldehyde, the previously frozen samples were decalcified in 0.1 N HCl. The samples were embedded in paraffin, and 6 µm sections were stained with the Masson-Goldner trichrome method.

The values are given as mean ± standard deviation (SD). Student's *t*-test was used to compare the densitometric and mechanical values between the study groups. Because of the ordinal measurement scale, non-parametric Mann-Whitney test was used to compare the BU and BF scores between the study groups. The statistical analyses were performed using the SPSS for Windows statistical package (SPSS Inc., ver. 7.5.1). Values of $P<0.05$ were considered statistically significant.

Results

The dogs tolerated the experiment well, and there were no infections or complications. The plates were broken in two cases treated with xenograft with BMP, and there was some loosening of screws and plates in all cases of both groups, indicating inadequate healing.

At the end of the study, no instances of bone union of the defect were seen in the cases treated with either pure xenograft or xenograft with BMP, the mean score for bone union being zero in both groups. There was some bone formation at the bone ends in all cases of both groups. Xenogeneic implants with BMP induced more bone formation than the implants without BMP, the scores being 1.0±0.7 and 0.5±0.6, respectively, but the difference was not statistically significant ($P=0.24$).

The xenogeneic implant, although demineralized, was faintly visible during the first few weeks. The implants, both with and without BMP, showed complete resorption in roentgenograms at 20 weeks (Fig. 1).

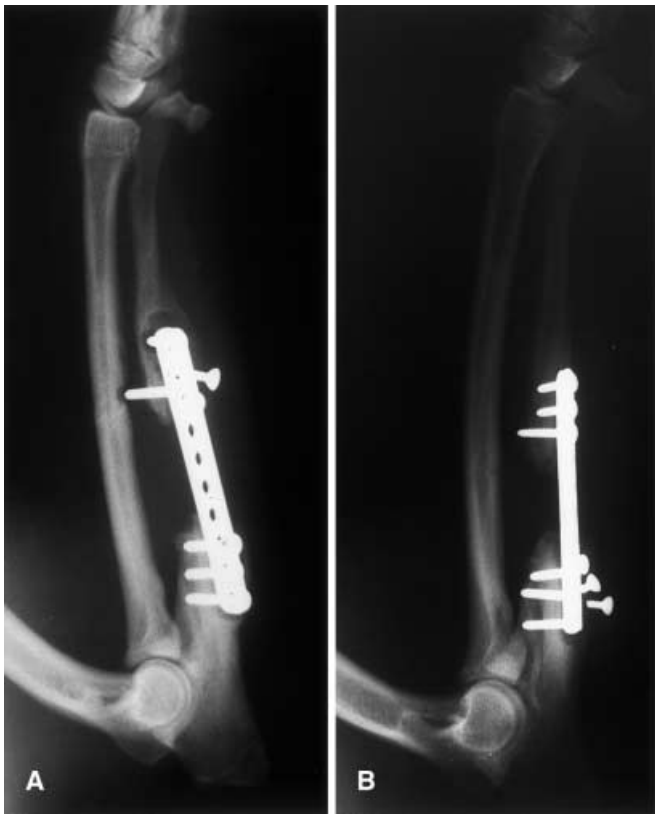

Fig. 1 Roentgenograms showing an ulnar defect at 20 weeks treated **a** with a pure xenograft and **b** with a xenograft with BMP. No complete healing can be seen in either case. Some bone formation at the bone ends is seen – slightly more in the case treated with a xenograft impregnated with BMP. Both implants appear completely resorbed at 20 weeks

Table 1 Maximal torque capacity (Nm) of dog ulnas after 20 weeks' healing of 2-cm tubular defects treated with pure xenografts and xenografts with BMP

Dog	Xenograft	Xenograft+BMP
1	0.66	1.00
2	0.20	0.48
3	0.56	0.15
4	0.12	0.33
5	0.14	0.93
6	0.14	0.47

There was a significant difference in the total BMC in the proximal ulna close to the defect in favour of the cases treated with xenograft with BMP (P=0.047). On the other hand, the total BMD of the distal ulna near the defect was lower in the group treated with xenograft + BMP implant (P=0.022). However, no significant differences in BMD, BMC, or CSA were seen between the groups in the implant area or at the bone-implant border.

In mechanical testing, all the bones of both study groups broke at the implant area, indicating a weak or absent bony union. The average MTC of the ulnas treated with a xenograft impregnated with BMP

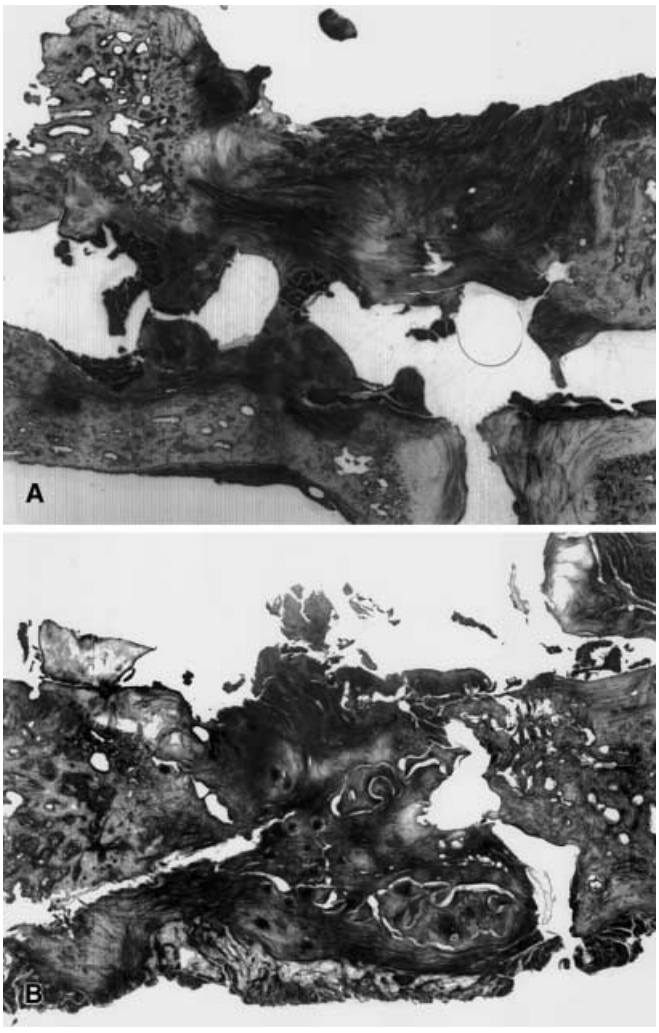

Fig. 2 Photomicrographs of histological sections of the ulnar defect areas at 20 weeks with **a** a pure xenograft, showing fibrotic tissue and some new bone at the bone ends, and **b** a xenograft with BMP, showing also some fibrosis, but more remodellation of bone. Masson-Goldner trichrome staining

(0.56±0.34 Nm) was higher than that of the ulnas treated with a pure xenograft (0.30±0.24 Nm), but the difference was not statistically significant (Table 1).

Fibrotic tissue was found in the implant area in all the cases treated with xenografts without BMP (Fig. 2a). There was more remodellation of the bone in the cases treated with xenografts with BMP. However, there was also fibrosis between the bone ends in all cases (Fig. 2b).

Discussion

Xenogeneic bone has been studied as an alternative to autogenous and allogenous grafts by many authors [3, 6, 7, 11, 13, 14, 19]. Salama [14] used xenogeneic bovine bone (Kiel bone), which was hydrogen peroxide extracted, fat solvent treated and dried with acetone, in various clinical situations in humans, including fractures, benign

bone lesions, and pseudoarthrosis. The results were satisfactory, and he reported no complications that could be attributed to the use of xenograft bone [14]. Mineralized xenogeneic, bovine-originated bone sintered by removing all organic material while retaining the bone microstructure, has been applied with moderate success in rabbit bone defects [6, 19] and in human edentulous ridge defects [3], although the resorption of these materials is obviously poor [19].

However, there is a lack of consensus on the suitability of xenogeneic demineralized bone matrix (DBM) for bone transplantation, even in lower-order animals [1]. Ripamonti et al. [13] showed that baboon and human DBM induced enchondral bone formation in athymic rats and baboons, but not in euthymic rats. Bone formation induced by human DBM in baboons suggests that intact bone matrices processed in this manner may not be species-specific, at least among primates [13]. Here, demineralized bovine cancellous bone was unable to induce new bone formation in canines. These species may be so distant phylogenetically that xenograft fails to be osteoinductive.

It has been suggested that BMP might enhance bone formation in combination with xenogeneic bone material. Hollinger et al. [7] studied the healing of critical-size cranial defects in non-human primates, using autografts and xenogeneic human antigen-extracted, autolyzed bone impregnated with bovine BMP. The autografts resulted in the greatest volume of new bone formation, but the antigen-extracted, autolyzed bone elicited a significantly greater response than either the bovine BMP derivatives or the controls. Minamide et al. [11] studied sintered xenogeneic bone with type I collagen and recombinant human BMP (rhBMP-2) in a rabbit spinal fusion model, and the results showed that xenogeneic bone with rhBMP-2 resulted in a higher fusion rate than autograft. In our study, we used a xenogeneic demineralized bovine bone either alone or with bovine-derived BMP, and found that added BMP seemed to increase bone formation, but failed to lead to a complete bony union. The immunological reaction with xenograft bone processed in the method we employed, possibly exceeds the effect of BMP.

The resorption of the implants was very rapid when evaluated roentgenologically. The resorption of the graft might have been too fast to enhance proper ossification, even with BMP. This is in line with some earlier findings, where the suitability of xenograft has been considered questionable because of the fast resorption [2].

It is concluded that native, partially purified BMP does not improve sufficiently the suitability of bovine demineralized xenograft as a bone substitute material for canines. Demineralized xenogeneic bone does not seem to be a feasible carrier material for BMP.

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