Tibial segmental bone defect treated with bone plate and cage filled with either xenogeneic composite or autologous cortical bone graft

An experimental study in sheep

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Summary
Tibia segmental defect healing in sheep were clinically, radiographically and histologically evaluated. Twelve young sheep aged four to five months were divided into two groups, G1 and G2. A 3.5 cm long segmental defect was created in the right tibia diaphysis with maintenance of the periosteum. The bone defects in both groups were stabilized with a bone plate combined with a titanium cage. In G1 the cage was filled with pieces of autologous cortical bone graft. In G2 it was filled with a composite biomaterial which consisted of inorganic bovine bone, demineralized bovine bone, a pool of bovine bone morphogenetic proteins bound to absorbable ultra-thin powdered hydroxyapatite and bone-derived denaturated collagen. Except for one G1 animal, all of them showed normal limb function 60 days after surgery. Radiographic examination showed initial formation of periosteal callus in both groups at osteotomy sites, over the plate or cage 15 days postoperatively. At 60 and 90 days callus remodeling occurred. Histological and morphometric analysis at 90 days after surgery showed that the quantity of implanted materials in G1 and G2 were similar, and the quantity of new bone formation was less (p=0.0048) and more immature in G1 than G2, occupying 51±3.46% and 62±6.26% of the cage space, respectively. These results suggest that the composite biomaterial tested was a good alternative to autologous cortical bone graft in this experimental ovine tibial defect. However, additional evaluation is warranted prior to its clinical usage.

Keywords
Bone defect, treatment, biomaterials, bone graft, sheep

Introduction
Bone grafting materials may enhance bone formation and healing in bone defects by three mechanisms: osteoconduction, osteogenesis, and osteoinduction (1, 2). Fresh autologous bone graft has all these properties but, due to restricted quantity and the need for donor site surgery with potential morbidity, the use of this type of graft can be inconvenient (2, 3).

An alternative treatment for large segmental defects is the allogeneic cortical bone graft, but it has several limitations, such as low osteogenicity, more intense absorption, immune-mediated rejection, and a risk of infectious disease transmission (3, 4).

All these problems have stimulated extensive research into the development of bone graft substitutes with osteogenic properties near to that of autologous bone. Basically there are two main types of bone graft substitutes available: osteoinductive materials that stimulate differentiation of new osteogenic cells by the active recruitment and induction of pluripotent mesenchymal cells; and osteoconductive materials that serve as an inert scaffold for the ingrowth of new bone (2, 3). Thus, demineralized bone matrix (DBM), inorganic bone and bone morphogenetic proteins (BMPs), alone or as a composite, have been proposed as bone graft substitutes (5, 6).

DBM is a biomaterial which is produced from allogeneic or xenogeneic bone by eliminating the mineral content with acid treatment. It mainly consists of type I collagen and traces of osteoinductive growth factors such as BMPs (6–8). DBM-enhanced bone formation seems to be associated with osteoconductive rather than osteoinductive capability (9, 10).

Inorganic bone is a natural hydroxyapatite, obtained by thermal deproteinization of cancellous bone granules which gives higher osteoconductive properties (11).

BMPs are the only bone growth factors that can induce differentiation of undifferentiated mesenchymal cells into chondroblasts and osteoblasts (4, 5). Native BMPs and recombinant BMPs, especially BMP 2, 4, and 7, are able to regenerate critical size bone defects in sheep, rodents, dogs, and primates when associated with a collagen, DBM, hydroxyapatite, and biodegradable polymer carrier (5–7). The efficacy of BMP may be affected by its concentration, carrier properties, hormones, growth factors, and the presence of target cells (9).

The treatment of segmental bone loss aims to reconstruct bone tissue as well as to normalize deficient limb function. The fixation method should be comfortable for the patient, allow bone regeneration, and provide sufficient rigidity to support weight-bearing during locomotion. This study evaluated clinical, radiographical, and histological aspects of the healing of segmental defects, surgically created in sheep tibia and stabilized with a bone plate and cage filled...
with either cortical autologous bone graft or composite biomaterial, which consisted of inorganic bovine bone, demineralized bovine bone matrix, a pool of bovine bone morphogenetic proteins (bBMPs) bound to absorbable ultra-thin powdered hydroxyapatite, and bone-derived denaturized collagen.

**Materials and methods**

This study was approved by the Ethics Committee of the Veterinary School of São Paulo State University. Twelve clinically healthy sheep initially weighing 21–38 kg (mean 30.9 kg), and aged four to five months old were equally and randomly divided into two groups (G1 and G2) with three males and three females in each group. They were housed in four pens (9 m²), and received sheep maintenance ration (Ovinotech Campo; Purina, São Paulo, Brazil), and hay and water *ad libitum*.

**Surgical procedures**

The ration was removed 48 hours before the surgical procedure, and the hay and water 24 hours previously. After premedication with midazolam (0.3mg/kg IV) and butorphanol (0.2 mg/kg IV), general anaesthesia was induced with ketamine (2 mg/kg IV) and maintained with halothane. The sheep was positioned in right lateral recumbency, and the right hindlimb was clipped, prepared, and draped using a sterile technique. A longitudinal skin incision was performed on the craniomedial aspect of the right tibia midshaft. The periosteum was incised longitudinally and carefully elevated circumferentially exposing the tibial diaphysis (Fig. 1a). A 3.5 cm segment of bone was removed from the midpart of the tibial shaft with the use of an oscillatory bone saw under irrigation with 0.9% saline solution (Fig. 1b). In both groups the bone defects were stabilized with a titanium bone plate (103 mm long, 10 mm wide, 2 mm thick, with six AO-like dynamic compression holes) combined with a titanium cage (35 mm long, 15 mm diameter) (Placa especial p/tibia c/BOB; Baumer, São Paulo, Brazil) (Fig. 3c). Both
the diameter and the distance between the holes of the cage were 2 mm long. In G1, the cage was completely filled with pieces of autologous cortical bone graft (0.5–1.0 cm long and 0.3–0.7 cm wide) which had been obtained from approximately 50% of the removed tibial bone segment using bone cutting forceps. In G2, the cage was completely filled with a composite lyophilized bone graft (GEN-tech; Baumer, São Paulo, Brazil) which contained inorganic bovine bone, demineralized bovine bone matrix, a pool of bBMPs bound to absorbable ultrathin powdered hydroxyapatite, and bone-derived denatured collagen agglutinant mixed with 1 ml of autologous blood. The plate was fixed to the bone with six titanium cortical screws (3.5 diameter) (Baumer, São Paulo, Brazil), three proximal and three distal to the cage (Fig. 3d). The internal fascia and peristomeum were mainly closed at the plate extremities with simple continuous sutures. However, the size of the cages did not allow total periosteal coaptation at the central area of the plate. The subcutaneous tissue was closed using simple continuous pattern and the skin incision using simple interrupted pattern. All of the sutures were inserted with 3.5 metric nylon monofilament.

Pre-and post-operative care

Enrofloxacin (2.5 mg/kg SC q24h) was administered immediately preoperatively and for five days postoperatively. Flunixin megamumine (2.2 mg/kg SC q24h) was administered for three days after surgery, and buprenorphine (0.01 mg/kg IM q8h) for five to seven days. The skin suture was removed 15 days after surgery.

Clinical and radiographic evaluations

Limb function was evaluated daily according to the following scoring system: 0 = normal ambulation, no lameness; 1 = slight lameness, toe touching; 2 = lameness, some toe touching, otherwise limb carried; 3 = lameness, limb carried (non-weight bearing) except when herded; 4 = lameness, limb carried (non-weight bearing) even when herded. Cranio-caudal and lateral radiographs of both tibias were obtained immediately after surgery and at 15, 30, 60, and 90 days post-operatively. Developed periosteal callus height (mm) at the proximal osteotomy site, mid portion of cage, and distal osteotomy site were measured by cranio-caudal radiography at 15, 30, 60, and 90 days after surgery. The data were subjected to an analysis of variance (ANOVA) followed by Tukey test using GraphPad Instat software. The differences were considered to be statistically significant at p<0.05.

Histological procedures

The animals were euthanatized 90 days after surgery and tibias were collected for histological evaluation. The bone specimens were measured and fixed in 10% phosphate buffered formalin pH 7 for 21 days. They were demineralized in 4.13% EDTA (Tritriplex I II – Merck®, Germany) adjusted to pH 7.2 with sodium hydroxide. The demineralizing solution was changed weekly. After bone demineralization, the cage was cut exactly at the junction with the plate, and the plate and screws were removed manually. The titanium cage was then slightly distracted, and the cut bone ends and the segmental defect area were removed en bloc. The demineralized specimens were dehydrated in ethanol, clarified in xylol and embedded in Histosec (paraffin + synthetic resin). Longitudinal semi-serial 5 µm thick sections were obtained, including the cut bone ends and proximal and distal parts of the segmental defect area, and transversal semi-serial 5 µm thick sections were obtained from the central area of segmental defect (Fig. 2). All of the sections were stained with haematoxylin-eosin.

Stereological analysis

For stereological analysis, 20 longitudinal semi-serial 5 µm thick sections with 1 mm intervals between the sections, including the cut bone ends and the proximal and distal parts of the segmental defects. Other 20 transversal semi-serial 5 µm thick sections (with the same space interval) from the central region of segmental defect were obtained for each animal. Ten stratified, random transversal (n=5) and longitudinal (n=5) sections per animal were selected by systematic sampling for stereological analysis.

The volume density (Vvi) or the fraction of titanium cage volume occupied by the graft, new bone, myeloid tissue and connective tissue were determined using a digital image analysis system consisting of a Zeiss Axioskop microscope, a Sony CCD-IRIS-RGB camera, and Kontron KS-300 software (Kontron Electronic GmbH) installed on an IBM computer. The images were captured using a 40X oil objective from 30 histological fields per section (300 fields per animal); they were selected by systematic randomiza-
The number of 30 histological fields per section had previously been assessed in a pilot study using the multiple $\chi^2$ sample homogeneity test with a probability level of 5%. The area occupied by each type of structure $(A_i)$ and the total area analyzed $(A)$ were determined from these images. Each type of structure's area density $(AA_i)$, which corresponded to its volume density $(V_{vi})$, was calculated according to the $AA_i = V_{vi} = A_i/A$.

Comparisons between group data were by Student's $t$ test using Sigma Stat–Jadel$^\text{™}$ a Scientific software for Windows; the significance level was set at 0.05. The volume density being a percentage of the normal distribution. Thus, for statistical parametric analysis application it was necessary to perform the arc sine transformation of the original data.

### Results

#### Clinical and radiographic evaluations

The surgical wounds healed without any complications. Seven to 10 days after surgery, all of the animals had a limb function score of 2. They all showed normal limb function 60 days after surgery, except for one G1 animal. The final body weight varied from 24 to 43 kg (mean 35.5 kg).

Direct measurements from radiographs showed that the amount of segmental bone defect, according to tibial length, was from 16.67 to 20.23% (mean 18.41%) in G1, and 15.70 to 20.47% (mean 18.24%) in G2. Plate bending (3° to 9°) was observed in the transition zone between the plate and the plate-cage construct, especially proximally, in four G1 animals and one G2 animal 15 days after surgery. There were not any further cases of plate bending observed at the later radiographic examinations. At the last evaluation 90 days after surgery, a difference was not observed in the length of treated and non-treated tibia in the same animal. The magnitude of tibial deviation in cases of plate bending ranged from 3° to 14° in the varus position.

Initial periosteal callus formation was observed in both groups, over the plate or cage, at the osteotomy sites 15 days after surgery. Periosteal callus production was more abundant 30 days after surgery, and remodelling and reduction were observed 60 and 90 days after surgery (Figs. 3, 4). The mean periosteal callus height (mm) was not statistically different between evaluation moments in the same group or between groups.

A proximal or distal radiolucent line was observed between cut bone end and cage in 4 G1 and 3 G2 animals 15 days after surgery. This line was absent in all animals at 90 days after surgery. Cage holes were visible in both groups at the radiographic examination immediately after surgery, but there was some radiopacity associated with filling materials. The holes were less visible at the other evaluation times. Plate or screw breaking was not detected. Also, the screws did not show any signs of bending or loosening.

### Histological results

Histology of the defects treated with cortical autologous bone graft (G1) 90 days after surgery showed signs of resorption of the old bone at the proximal and distal cut bone surfaces, and replacement by trabecular new bone and the regions of the old medullary canals occupied by myeloid tissue.

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*a* Sigma Stat–Jadel™, Jadel Corporation, Chicago, IL, USA.

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![Fig. 4](image-url) Craniocaudal radiographs of the tibia defect treated with bone plate combined with a titanium cage filled with biomaterials (G2): (a) immediately after surgery and at; (b) 15; (c) 30; (d) 60; and (e) 90 days after surgery.

![Fig. 5](image-url) (a) Panoramic photomicrograph of the mid area of a G1 (a) and G2 (b) bone defect 90 days after surgery. (a) Notice the site of cage holes (arrows) filled with newly formed bone tissue covered by connective tissue, and the central area filled with bone tissue (BT) and loose connective tissue (CT). (b) Observe the site of cage holes filled with newly formed bone tissue covered by connective tissue, and the central area with inorganic bone (asterisks) surrounded by newly formed bone (NB). HE, 8X.
and some autograft fragments associated with new bone. The inside of the titanium cage or defect space (Figs. 5a, 6a) was filled with large fragments of cortical bone graft exhibiting reabsorption areas by osteoclasts (Fig. 6c) and other areas intimately associated with new bone. The distinction between the new bone tissue and grafted autologous cortical bone was possible because the large graft particles of cortical bone graft exhibited lamellar cortical structure, numerous empty osteocytic lacunae and cement lines between the particles and new bone. On the other hand, the newly formed bone at 90 days showed non-lamellar woven bone organization and a higher number of osteocytes (Fig. 6b, c). Also, a few small areas of chronic inflammatory process and extensive areas of loose connective tissue were seen (Fig. 6a). The titanium cage holes were filled with trabecular bone tissue with several spaces occupied by loose connective tissue and some myeloid tissue (Fig. 5a). Next to the internal surface of the cage, the new bone was covered with fibrous connective tissue that isolated the bone tissue from the metal surface (Fig. 6d). The internal new bone connected via cage perforations to a thick external layer of new bone had completely enveloped the cage.

The defects treated with composite graft (G2) did not show any signs of inflammatory process. The cut bone ends did not display any signs of reabsorption and the regions of the old medullary canals filled with myeloid tissue and some particles of the biomaterial surrounded by newly formed bone. There were only inorganic bone particles in the defect space wrapped by abundant new bone tissue (Fig. 5b). The space inside the titanium was completely filled with newly formed bone that fused on to the cut bone ends. The new bone had a compact aspect with some nutrient canals surrounded by concentric lamellar arranged bone matrix (Fig. 7a). The pores of the inorganic bone particles were filled with newly formed bone, but they did not exhibit signs of reabsorption (Fig. 7b). The new bone inside the cage holes (Fig. 7c) and next to the internal surface (Fig. 7d) was similar to that observed in G1.

**Stereological results**

The quantities of remaining graft material and new myeloid tissue in G1 and G2 were not significantly different at 90 days (Fig. 8). However, the volume of newly formed bone with the cage space in G1 (51 ± 3.46%) was less than in G2 (62 ± 6.26%) (p=0.0048).
Conversely, the volume of connective tissue G2 was less than in G1 (p<0.05).

**Discussion**

Gugala and Gogolewski (12) determined the critical defect size in adult sheep tibial diaphysis as a 4 cm osteoperiosteal segmental defect. In addition, Den Boer et al. (13) reported that a 3 cm segmental defect created in adult sheep tibial diaphysis should not be considered a non-union model if the periosteum is maintained, because such a bone defect may be reconstituted. The defect used in our study cannot be considered of critical size, since it was 3.5 cm long, the periosteum was maintained and the sheep were young.

The bone plate combined with titanium cage was partially wrapped with remaining periosteum, since in young sheep the periosteum is usually thick and easily identified. The findings of some other experimental studies suggested that the periosteum can be considered a natural membrane to guide bone regeneration, but it is necessary to place some type of implant, such as our titanium cage, within the defect to prevent collapse (14, 15). The latter was represented, in the report presented herein, by the perforated titanium cage. Since mean periosteal callus heights (mm) were not statistically different between groups, it seemed that the periosteal response to the implanted cage was independent of the type of material contained within the cage.

The bone plate was developed to maintain alignment and stability of the osteotomized tibia, similar to a bridge plate, and at the same time the plate retained the titanium cage within the bone defect. Titanium cages are frequently used in human patients to obtain spinal fusion or spinal reconstruction (16, 17). However, few reports have evaluated titanium cages in the treatment of segmental defects of long bones (18–20). In these other reports, the cages were used with other methods of immobilization, including intramedullary pins (20), interlocking nails (19), or bone plates (18).

The titanium cage was attached to the plate in order to avoid displacement or migration. It should be pointed out that this is clinically inconvenient, since the plate and cage size must be developed according to the limb length and segmental bone defect dimensions, and the radiopacity of these implants impedes post-surgery radiographic evaluation of bone formation. Although our observations of progressive diminution of radiolucency within cage holes at sequential postoperative evaluations may be considered to be indicative of bone formation, we do not consider these observations to be accurate enough in order to be able to establish a comparative pattern between groups.

In 41.6% of the animals, plate bending (3° to 9°) was observed in the transition between plate and cage which indicates a difference in mechanical resistance in this area. This problem was not observed by authors who used the cage not connected to the fixation method (18–20). Although the central section of the plate had a connected cage and no hole, the plate thickness may not have been sufficient enough in order to support the forces. In addition, the limbs were not protected by external coaptation and at around seven to 10 days after surgery the animals started to bear weight on the operated limb inducing load on the implant.

Bending of the plates was not a complication beyond 15 days after surgery, despite the increasing body mass of the sheep and weight bearing on the osteotomized limb during the course of the experiment. The bone formation seen radiographically at this time probably helped in strain distribution. Also, when treating 2.5 cm osteoperiosteal segmental defect in sheep femur using a bridge plate (30 mm central section) and eight screws, Gerhart et al. (21) observed a 30° bending of the plate in two animals. This bending occurred at the screw-hole closest to the proximal edge of the defect and was non-progressive. In contrast to our experiment, those authors also observed plate fixation loosening and failure in three sheep.

The radiolucent line at the transition between plate and cage was observed 15 days after surgery in all of the animals, which was probably associated with bone resorption due to heat necrosis and trauma induced by osteotomy, and not to implant failure.

Although fresh autologous cancellous bone is the most commonly used graft material due to its osteogenic, osteoconductive, and osteoinductive properties (3), we elected to use autologous cortical bone graft in G1 as our “control” because cortical bone fragments can be used clinically as graft in the multifragmented fracture with severe bone loss. Small pieces of compact bone implanted in a recipient site give more exposure to vascular canals and allow larger fragment revascularization (22). We used larger bone fragments, since small particle cortical morselized graft is considered to be ineffective because mechanical cellular destruction during grinding may diminish the osteoinductive activity (23). Ninety days after surgery, histology showed the defect area filled
with fragments of autologous bone graft associated with newly formed bone, which suggests that the size of the cortical bone fragments did not interrupt bone formation.

The composite bone graft used in G2 was developed by the Brazilian national industry specifically for use in human patients (24). Histology at 90 days after surgery in G2 did not show any signs of reabsorption in cut bone ends and predominance of lamellar and remodeled bone, which suggests a more mature bone than in G1. There is evidence that a combination of biomaterials may result in a synergic effect for bone neoformation (5, 6). Some hypotheses will be discussed regarding different components in the composite bone graft.

The processing of bovine bone may result in different biomaterials with specific properties. Demineralized bone presents osteoinductive and osteoconductive properties (9, 10, 25, 26, 27), while sintered inorganic bone matrix are osteoconductive (28).

However, due to its high collagen content, demineralized bone matrix is readily absorbed and its effect was related to the BMP content (25, 27). In this study, the composite tested also contained a pool of bBMPs that would be expected to enhance the osteoinductive effect (25). The inorganic bone matrix present in the composite was not absorbed until 90 days after surgery, it did not produce a foreign body reaction and some of the particles were wrapped by new formed bone. These results suggest that the combined properties of each biomaterial present in the composite contributed to the acceleration of bone repair, which possibly explains the predominance of remodeled new bone.

However, clinical and experimental results using this pool of bone biomaterials are controversial. Lima (29) observed by histomorphometric analysis that the area of new bone was larger in rabbit radius fractures which were treated with bBPMs pools than in controls 60 days after surgery. On the other hand, Costa Filho et al. (30) detected an inhibitory effect of pool bBMPs associated with inorganic bovine bone used to fill titanium cylinder prototypes implanted in rabbit tibia. Also, Guimarães et al. (31) did not observe any additional effect when pool bBMPs were associated with absorbable hydroxypatite carrier, lyophilized demineralized bone matrix, and membrane barrier in treating human patients with bone defects. BMP bone induction capability is dependent on preparation type, source, and purity, BMP availability, and dose (3, 26, 29), but there was not any available information on the purity and specificity of bBMP.

These results suggest that the composite biomaterial which was tested proved to be a good alternative to autologous cortical bone graft in this experiment ovine tibial defect. However, additional evaluation is warranted prior to its use in clinical cases.

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References


**BOOK REVIEWS**

S. Lindley, M. Cummings:

**Essentials of Western Veterinary Acupuncture**

191 pp.

Blackwell Publishing, 2006

The authors of ‘Essentials Western Veterinary Acupuncture’ present a concise and very readable overview of veterinary acupuncture. Once one starts to read this book it is almost impossible to put it down.

The book is divided into three parts plus an Introduction. The latter gives the reader an honest look at acupuncture. The authors aim to describe the neurophysiological aspect of acupuncture, as opposed to taking the traditional approach to the subject. They immediately point out that this is not a book from which to learn needling techniques.

Part one reviews acupuncture safety, the public perceptions of acupuncture, a historical overview of the evolution of the subject and the neurophysiological of pain, and how acupuncture can control it. The authors suggest that the veterinary profession will be more likely to accept acupuncture if researchers use an evidence-based approach.

Part two further explores the use of acupuncture for acute and chronic pain, visceral pain and non-painful conditions. One chapter is devoted to musculoskeletal pain that excellently describes myofascial ‘trigger points’, including the examination of a muscle for the ‘trigger points’ and the treatment and prognosis of the condition under care.

Part three describes the practical aspect of acupuncture, including information about needles, the restraint of animals for treatment purposes, assessment and response to the treatment. And, if one is so inclined, the prospects of starting an acupuncture clinic.

Finally, electroacupuncture techniques are presented as yet another modality to be considered, as well as trans-cutaneous electrical nerve stimulation and low intensity laser therapy.

The authors suggest that more evidence-based medicine is required in veterinary acupuncture. They strive for a safe approach to the subject and the information is practical, straightforward, honest and will entice an individual to learn more about veterinary acupuncture and, if one is already practicing the subject, it will complement what the veterinarian already knows.

This book is highly recommended. It is easy to read, and is not a typical textbook, and can be read in several sittings.