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Incorporation of vancomycin-supplemented bone allografts

Radiographical, histopathological and immunohistochemical study in pigs

Martín A Buttaro[1], Alejandro M González Della Valle[1], Laura Piñeiro[2], Esteban Mocetti[2], Ana A Morandi[2] and Francisco Piccaluga[1]

[1]Institute of Orthopaedics "Carlos E. Ottolenghi", [2]Pathology Department, Hospital Italiano de Buenos Aires.
Correspondence: Martin A. Buttaro, Institute of Orthopaedics "Carlos E. Ottolenghi", Hospital Italiano de Buenos Aires, Potosí 4215 (C1199ACK), Buenos Aires, Argentina. cadera@hospitalitaliano.org.ar
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ABSTRACT **We compared the incorporation of bone allografts with or without vancomycin in tibial defects of 18 pigs. High-quality radiographs, histological examination, immunological expression of metalloproteinase-13 (MMP-13) and transforming growth factor-beta 2 (TGFß$_2$) indicated that there was no significant difference in bone allograft incorporation between up to 220 times the MIC (minimum inhibitory concentration) in bone allografts with 1 g of vancomycin in each 300 g of allograft or without this supplement.**

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Cancellous bone has been used as an antibiotic carrier since De Grood mixed it with penicillin to fill bone defects in 1947. Recent experiments with cancellous bone as a delivery vehicle for vancomycin in vitro and in vivo have shown high initial peak concentrations of antibiotics in the surrounding medium, followed by low levels for long periods (Witso et al. 1999, 2000, Winkler et al. 2000). However, high concentrations of vancomycin can substantially reduce osteoblast replication and even cause cell death (Miclau et al. 1993, Edin et al. 1996).

To determine whether supplements of vancomycin impair the incorporation of bone allografts, as compared to allografts without supplements, we implanted such grafts in healthy pigs.

Animals and methods

All procedures involving the laboratory animals were approved by our hospital ethics review board.

18 male Yorkshire immunized adult healthy pigs (mean weight 25 kg) were divided into two groups: 9 had a vancomycin-supplemented bone allograft and 9 a bone allograft without antibiotics. Anesthesia, induced with a mixture of 200 mg ketamine, 1 mL 1‰ atropine and 10 mg diazepam, was maintained with a 5% halothane oxygen mixture during the surgical procedures.

Surgical technique

The experiments were done on 2 pigs at the same time under sterile conditions (two surgical teams), each specimen from a different group (V+, V–).

Via a longitudinal approach to the middiaphysis of each right tibia, an identical quadrilateral unicortical defect (30 mm × 10 mm) was created in the anterior cortex of each bone, using a trephine inserted into guide holes. All soft tissue was removed from the medullary canal (Figure 1).

We made cortical windows in the proximal metaphysis of each right humerus, harvested 6 g of cancellous bone graft, and weighed them with a digital precision scale (Acculab V, Danvers, MA, USA; 1200/g, 0.1 g margin). The humeral defects were filled with bone wax (Ethicon, John-

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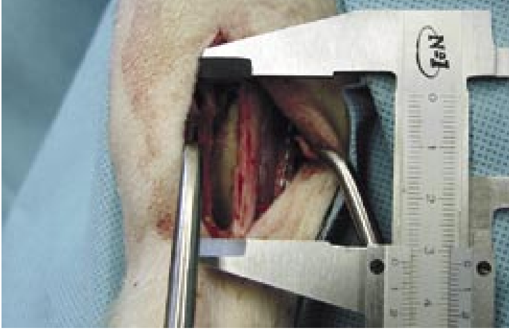

Figure 1. A quadrilateral unicortical defect (30×10 mm) was created in the anterior cortex of each tibia of the 18 pigs.

son & Johnson, Somerville, NJ, USA). The bone graft, corresponding to a V– group specimen, was manually fragmented into 0.4–0.6 mm pieces and mixed with 20 mg of powdered vancomycin (Lilly Indianapolis, IN, USA). At the same time, the bone graft, belonging to a V+ group specimen, was manually fragmented in the same way and placed in the defect of the V– group specimen. After mixing for 15 minutes, the morselized vancomycin-supplemented bone allograft was placed in the window of the V+ group specimen. No fixation was used and the wounds were closed. Immediate weight bearing was allowed. All 18 pigs survived. There were no intraoperative fractures, infections or bone graft dislodgement.

The pigs were killed with an intravenous injection of 5 mL KCl (15 meq) 30, 60 and 90 days postoperatively, as follows: at 30 days, 3 in the V+ group and 4 in the V– group, at 60 days, 3 in the V+ group and 3 in the V– group; 90 days, 3 in the V+ group and 2 in the V– group.

The tibias were harvested immediately the animals were killed.

Protocol for radiographs

After dissection of the tibias, radiographs were taken in the lateral view, using a modern X-ray unit (Multix, Siemens, Wendelsheim Germany), high resolution film (Kodak, Rochester, NY, USA), ma 7.1, kv 56, and a focus-film distance of 115 cm.

We used a double-blind classification: grade 1–complete absence of bone allograft; grade 2–bone allograft alone in the defect and no organization or evidence of cortical reconstruction in the medullary canal; grade 3–bridges of organized trabecular bone without peripheral callus; and grade 4–cortical reconstitution with compact bone and peripheral callus.

In the statistical analysis of the V+ and V- groups, we refer to grades 1 and 2 as low, and the others as high grades.

Protocol for histopathological examination

3 clearly distinguishable 0.3–0.5 cm transverse sections were taken from the area of the defect in each tibia. Specimens were routinely processed and embedded in paraffin in an automatic Citadel 2000 Wax Bath (Shandon, Pittsburgh, PA, USA) processor and 5 micron microtome sections were obtained from each sample. These samples were stained with hematoxylin & eosin (HE) and Masson's trichrome.

Microscopic examinations were done independently by the three pathologists in an optic microscope at 100× or 400×, depending on the parameter analyzed. For the statistical analysis, 10 areas in different microscopic fields were examined as regards each variable (parameter) in all samples from each animal.

The microscopic parameters evaluated were:

1. Necrosis. Extension (per 100× field) in the repair bone callus was classified as grade 1 – no necrosis; grade 2 – isolated areas of necrosis or moderate extension of necrosis.

The location of necrosis in the repair bone callus was classified as 1 – absent, 2 – central and 3 – central and peripheral.

2. Vascular ingrowth (neovascularization). This parameter, evaluated with HE and Masson's trichrome stains, was quantified as the sum of small capillary vessels per 3 fields at 400× magnification. All the repair bone callus in the samples was analyzed and 10 examinations were recorded.

3. Inflammation per 100× field was classified as 1–absent, 2–present. The type of infiltrate was specified (mononuclear, plasmocytic or polimorphonuclear). The presence of a granulomatous reaction was also evaluated (grade 1 – absent; grade 2 – present).

4. Reparative fibrosis in the repair bone callus was classified as grade 1 – mild, grade 2a – moderate and grade 2b – extensive) per 100× field. For the statistical analysis, we grouped grades 2a and 2b as grade 2.

5. Bone/cartilage distribution in the repair bone callus. The following parameters were evaluated as percentages of the repair area by endochondral ossification process (Masson's trichrome and HE samples): uncalcified cartilage, calcified cartilage, inmature bone, mature bone.

These variables were analyzed at the same time in 10 100× fields in the repair bone callus of each animal. Each 100× field was quantified with a grid consisting of 100 square areas (10×10). 100% of a given parameter—i.e., mature bone—was equivalent to the whole 100× field of 100 square areas entirely represented by mature bone. In the statistical analysis, 1% was considered equal to 1 square area of the field grid and 100% was considered equal to 100 square areas with a magnification of 100× in each observation of a given variable.

In the statistical analysis, the percentage of mature bone (number of square areas per 100X field) in each observation of each animal was regarded as indicating adequate repair and ossification. The findings in each animal were divided into two groups: mature bone ≥ 70% per 100× field, and mature bone < 70% per 100× field.

The repair bone callus was also analyzed in its entirety, using Vigorita's (1999) descriptive classification: a. provisional callus, b. hard callus, and c. remodeled callus.

6. Periosteum and subperiosteal bone. The percentages of trabecular and compact bone were classified in every sample per 100× field.

7. Bone resorption in bone callus. This parameter, analyzed at 400× magnification was determined by calculating the average number of osteoclasts per field.

Protocol for immunohistochemical examination

Two paraffin-embedded serial sections of 5 μm from the repair bone callus were mounted on positively-charged slides (Silane, Sigma, MO, USA). We selected the sections by HE staining.

The tissues were deparaffinized in 2 baths of xylene, treated consecutively with 100° alcohol and 96° and then rehydrated with distilled sterile water. Antigen retrieval was done with citrate buffer at pH 6 (Citra Biogenex, CA, USA).

Endogenous peroxidase blocking was performed with 10% hydrogen peroxide in PBS, pH 7.2, for 20 min. The slides were then washed twice in distilled water and rinsed twice in PBS, pH 7.2 + Triton X-100 (0.05%). The slides were incubated in goat serum (Biogenex, CA, USA) as protein block, and immediately afterwards, the serum was discarded. They were incubated with anti-metalloproteinase-13 (MMP-13) primary antibody (Cat. MAB3321, Chemicon, CA, USA) and anti-$TGFß_2$ (V) (Cat. Sc-90, Santa Cruz Biotechnology, CA, USA), using separate serial slides for each animal, in a humidity chamber (dilution 1:100 for both antibodies). Then the slides were rinsed twice in PBS/Triton and immediately incubated with 1:20 biotinylated secondary antibody (Multilink, Biogenex, CA, USA). After two more PBS /Triton washings, the slides were incubated with streptavidin-HRP label 1:20 (Label, Biogenex, CA, USA) and developed with DAB substrate + PBS buffer + hydrogen peroxide under a microscope for examination. We counterstained the slides with hematoxylin (Biopur, AR) and mounted them with synthetic medium.

The immunostainings were scored, using the following parameters:

1. Number (and %) of osteocytes, osteoblasts, osteoprogenitor cells and chondrocytes with TGFß2 cytoplasm positivity in the repair bone callus area per 50 cells (considered 100%) seen in every 100× field.
2. Number (and %) of hypertrophic chondrocytes and osteoblasts with MMP-13 cytoplasm positivity in the repair bone callus area per 50 cells (considered 100%) seen in every 100× field.

Statistics

In each animal, we made 10 observations of every histological or immunohistochemical classification. Continuous variables were expressed as means + standard deviation (SD), unless otherwise stated, and compared with the Mann-Whitney test. To ensure that the test for independent variables was not incorrectly used, all variables were also analyzed with the general linear model for repeated measures. Categorical variables were compared with the Chi Square test, and radiographical grades with Fisher's exact test. Differences were considered significant when the p-value was less than 0.05.

Table 1. Radiographic findings (higher grades mean better repair). Number of animals in each group

Grade/group	V+	V–
Low (1 and 2)	3	2
High (3 and 4)	6	7

p = < 0.598
V+ Vancomycin-supplemented allograft

Table 2. Histopathological results for dichotomic variables. Number (%) of fields with the variable. Total number of fields per group (V+, V–) analyzed = 90. Fields per animal = 10

Variable (grade 2)	V+	V–	P-value [a]
Necrosis	18 (20)	22 (24)	0.6
Inflammation	12 (13)	9 (10)	0.6
Reparative fibrosis	32 (36)	37 (41)	0.5
Granulomatous reaction	5	5	1

[a] Chi [2]
V+ Vancomycin-supplemented allograft

Table 3. Histopathological results. Vascular ingrowth (neovascularization). Number of small capilary vessels per 3 fields at 400x. Mean (SD)

Days	V+	V–	P-value [a]
30	17 (4.3)	17 (4.7)	0.9
60	14 (4.8)	15 (3.6)	0.4
90	8.7 (1.5)	8.6 (1.5)	0.9

[a] Mann-Whitney
V+ Vancomycin-supplemented allograft

Table 4. Number (%) of fields with 70% mature bone or more. Total fields per group (V+, V-) analyzed = 90. Fields per animal = 10

Days	V+	V–	P-value [a]
30	9 (30%)	10 (25%)	0.8
60	10 (33%)	10 (33%)	
90	30 (100%)	20 (100%)	_

[a] Mann-Whitney
V+ Vancomycin-supplemented allograft

Results

We found no significant differences within groups (fields in each pig) in the general linear model for repeated measures analysis of any of the variables analyzed (vascular ingrowth, mature bone, TGFß$_2$ positive cells, and MMP-13 positive cells). The findings are therefore shown, using the Mann-Whitney tests.

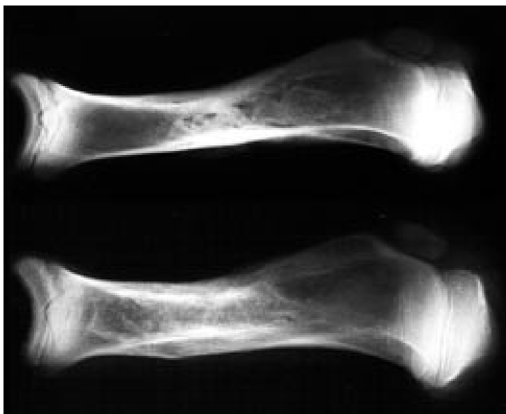

Figure 2. Complete reconstitution of the cortex at 90 days (upper figure: allograft without Vancomycin at 90 days, lower figure: Vancomycin-supplemented bone allograft at 90 days).

Radiography

We found no statistically significant difference between the groups in bone healing on the radiographs (Table 1).

At 30 days, a pattern consisting of organized trabecular bone had bridged the defects and no peripheral callus was seen. At 60 days, partial reconstitution of the cortex with compact bone and partial resorption of the allografts had occurred. At 90 days, both groups showed complete reconstitution of the cortex (Figure 2).

Histopathology

We found no statistically significant differences in the rate of graft incorporation between the groups at 30, 60 and 90 days in any of the parameters studied (Tables 2, 3 and 4).

We also found no statistically significant differences between the groups at 30, 60 and 90 days after surgery as regards the extension of necrosis and its location, neovascularization, inflammation, reparative fibrosis and granulomatous reaction in the repair bone callus.

At 30, 60 and 90 days postoperatively, the groups were similar concerning distribution of

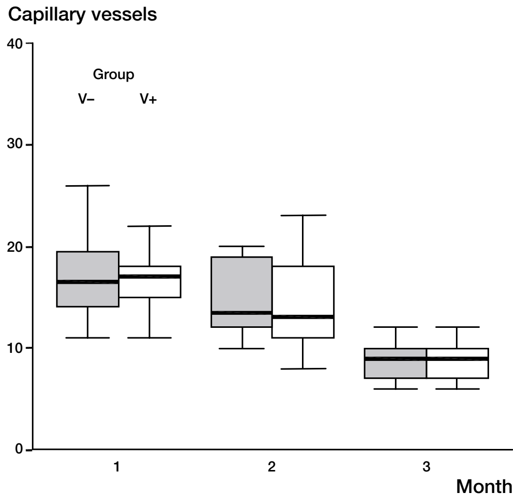

Figure 3. Vascular ingrowth in V+ (Vancomycin-supplemented) and V– groups at 30, 60 and 90 days (Box Plot).

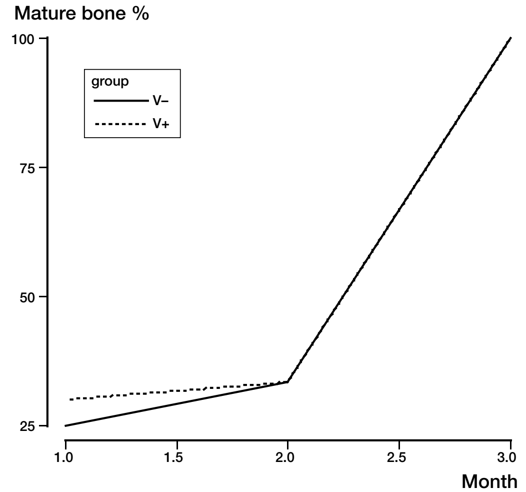

Figure 4. Percentage of mature bone in V+ (Vancomycin-supplemented) and V-groups at 30, 60 and 90 days.

bone/cartilage (Table 4 and Figure 3). Thus the percentages of subperiosteal trabecular or compact bone and bone resorption at the repair bone callus were about the same at the 3 examinations (data not shown).

However, the amount of necrosis, vascular ingrowth, granulomatous reaction and reparative fibrosis were greater in the 30-day specimens than in the 90-day ones. We regard this as a normal response to physiological bone repair and maturation.

Vascular ingrowth was seen in all allografts. The presence of vessels in the vancomycin-supplemented allografts at 30, 60 and 90 days was similar to that in the allografts without vancomycin (Table 3 and Figure 4).

At 30 days, the bone defects had more bone density in the peripheral zones. The same histological changes in bone graft incorporation occurred in both groups. It consisted of a soft repair bone callus characterized by granulation tissue with a moderate amount of collagen, immature cartilage and reticular bone tissue (Figure 5A and B). In both groups at 60 days, defects were bridged by a hard repair bone callus with reticular, trabecular, partly remodeled bone and small quantities of cartilage. No granulation tissue was seen in any of the groups (Figure 5 C and D). In both groups at 90 days the mature callus had remodeled, cartilage or granulation tissue as seen (Figure 5 E and F).

Immunohistochemistry

We detected no statistically significant differences between the groups in the expression of $TGFß_2$ in osteocytes, osteoblasts, osteoprogenitor cells and chondrocytes in the repair bone callus at 30 and 60 days (Table 5). A homogeneously positive expression of this growth factor was seen in osteocytes in the repaired bone, and in osteoblasts and chondrocytes of the endochondral ossification area in the repair bone callus (Figure 6).

At 90 days, the expression of $TGFß_2$ differed between the groups ($p = 0.017$)—i.e., the staining scores were higher in the vancomycin group.

Immunohistochemical analysis showed no statistically significant differences between the groups in the expression of MMP-13 in hypertrophic chondrocytes and osteoblasts in the repair callus at each time point (Table 6). The expression of this proteolytic enzyme was marked positive in several areas of the extracellular matrix, and extensive in the 30-day specimens (Figure 6B). This extracellular matrix proteinase stained more intensely in hypertrophic chondrocytes near the site of ossification.

Discussion

We found no difference in the healing of bone allografts with or without vancomycin used to treat

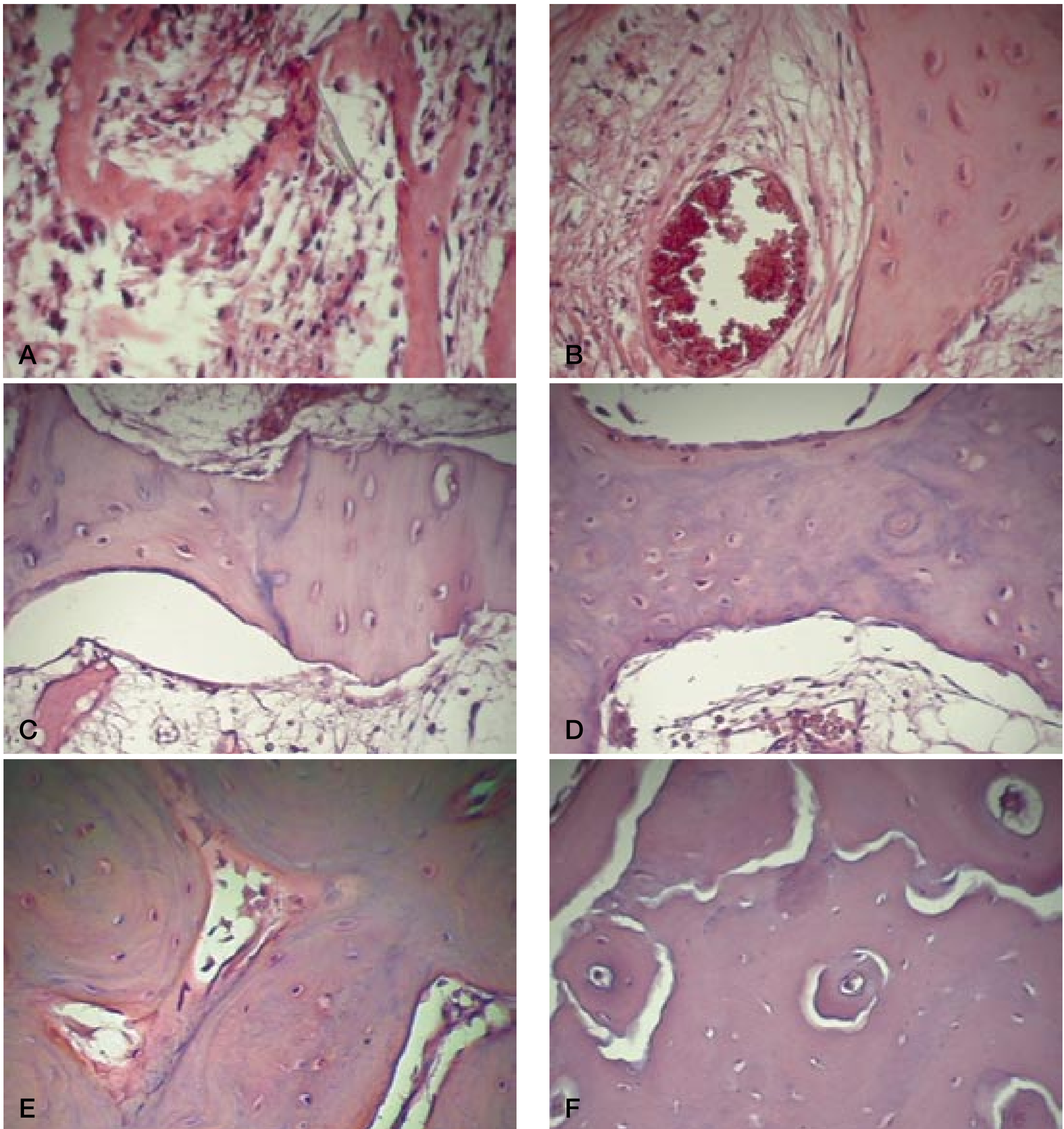

Figure 5. Histologic progression of bone allograft incorporation with and without Vancomycin at 30, 60 and 90 days. Left column: Vancomycin-supplemented bone allograft group (V+). Right column: non supplemented bone allograft group (V–) (Hematoxilin-eosin 200x).

Table 5. Immunohistochemical results. Number of TGFß$_2$-positive cells per 100x field. Mean (SD)/mean rank

Days	V+	V–	P-value [a]
30	31 (5.5) / 35	31 (5) / 36	0.9
60	21 (12) / 29	23 (9.6) / 32	0.4
90	34 (6) / 22	34 (6) / 31	0.02

[a] Mann-Whitney
V+ Vancomycin-supplemented allograft

Table 6. Immunohistochemical results. Number of MMP-13-positive cells per 100x field. Mean (SD)/mean rank

Days	V+	V–	P-value [a]
30	28 (12) / 34	29 (9) / 36	0.7
60	16 (13) / 34	15 (18) / 27	0.1
90	16 (15) / 26	34 (6) / 25	0.8

[a] Mann-Whitney
V+ Vancomycin-supplemented allograft

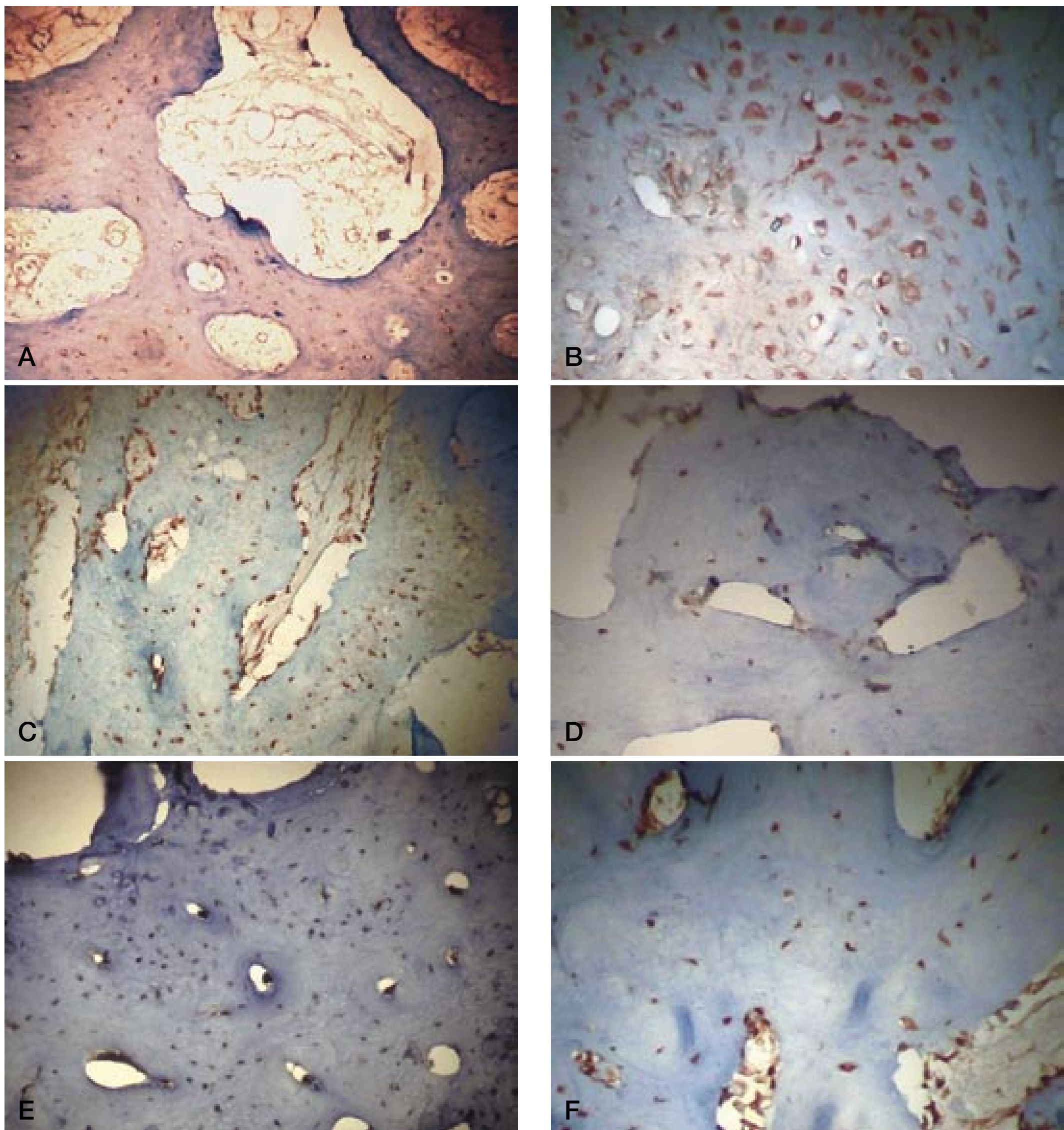

Figure 6. Immunohistochemical progression of bone allograft incorporation at 30, 60 and 90 days. Left column: homogeneously positive expression of of $TGFß_2$ in osteocytes, osteoblasts and chondrocytes in the repair bone callus in both groups at each period of time (Hematoxilin-eosin 100X). Right column: differences in the expression of MMP-13 in both groups observed at each time point (Hematoxilin-eosin 200x).

tibial defects in pigs, as assessed on radiographs or histological or immunohistochemical examinations. The use of cancellous bone to act as a carrier for vancomycin has been reported in vitro and in vivo (McLaren 1989, Miclau et al. 1993, Witso et al. 1999, 2000, Winkler et al. 2000). Initially, the concentrations of antibiotics in the surrounding medium are high; they then fall to lower levels which persist for long periods.

The incorporation of antibiotic-loaded bone grafts was first studied by Gudmundson (1971), who showed that the presence of tetracycline locally markedly inhibited bone graft incorporation. Gray and Elves (1981) reported that chloramphenicol and methicillin had adverse effects on osteogenesis. On the other hand, Petri (1984) found in histological studies of pigs, uneventful healing of fresh frozen bone allografts containing

cephalothin and tobramycin. Lindsey et al. (1993) also noted that tobramycin does not affect the normal healing features of cancellous bone autografts in dogs.

Whiteside (1994) evaluated the use of antibiotic-soaked bone grafts in cementless reconstruction of a previously infected knee arthroplasty. However, hardly any reports are available on the use of antibiotic-impregnated bone grafts in patients requiring reconstructive procedures with morselized bone.

We studied the expression of MMP-13 beause this collagenase plays an important role in bone healing, when a large amount of cartilage forms. This process requires a change in the extracellular matrix component from type II to type I collagen (Cawston 1998, D'Angelo et al. 2000, Hembry et al. 2001). In situ hybridization shows that this transition starts in the cells of the periosteum on day 1 and MMP-13 may initiate the process, and can easily cleave type II and type I collagen (Yamagiwa et al. 1999). Moreover in the reparative and remodeling phases, both hypertrophic chondrocytes and immature osteoblastic cells in the repair callus strongly express MMP-13 (D'Angelo et al. 2000, Uusitalo et al. 2000). This metalloproteinase also initiates the degradation of cartilage matrix, and causes resorption and remodeling of the callus (Yamagiwa et al. 1999).

The transforming growth factor-beta superfamily of growth factors is thought to play important roles in long-bone (endochondral) formation, fracture healing, and osteoblast proliferation (Rosier et al. 1998). An in vitro study has shown that the production of MMP-13 by human osteoarthrotic chondrocytes depends on the physiologic state of the cell, and that TGFß2 can trigger the procuction of MMP-13 (Tardif et al. 1999). Locally-produced TGF-ß regulates fracture repair in humans from an early (mesenchymal proliferation) stage to the stage of remodeling of woven bone. For this growth factor, animal models accurately reflect bones in human repair (Andrew et al. 1993).

In our study, we found no statistically significant differences at 30, 60 or 90 days after surgery between allografts with or without vancomycin in the expression of MMP-13 and of $TGFß_2$. This observation suggests that these proteins undergo similar changes during osteogenesis. MMP-13 showed transient variations in both groups, with a peak in slight expression peak at 30 days, which then declined at 60 and 90 days. These variations may be due to the involvement of MMP-13 in the early stages of cartilaginous matrix degradation, which is then replaced by bone matrix. On the other hand, TGF_2 values were similar at 30 and 90 days in both groups, which suggests that the expression of this pro-osteogenic growth factor remains at the same level during bone healing. Although these findings were not quantitative, they confirm those of others showing $TGFß_2$ expression during bone healing (Joyce et al. 1990, Izumi et al. 1992, Andrew et al. 1993). The differences in $TGFß_2$ expression between the groups at 90 days apparently do not affect bone healing, as judged by the absence of statistically significant differences in the histological parameters of bone repair and similar radiographic findings in both groups at this stage.

Further experiments in this and other models, including a long-term histological evaluation of the repair bone callus in larger groups of animals may explain these findings.

We used vancomycin in this study because it is one of the most clinically and experimentally evaluated antibiotics used locally (Edin et al. 1996)). It is available as a powder, is an effective bactericidal against most gram-positive organisms, including methicillin-resistant *Staphylococcus aureus,* has low resistance, causes few allergies, is heat-stable and water-soluble (Sande and Mandell 1985). It has been shown that local levels of 10,000 μg/mL of this antibiotic induce osteoblast death (Edin et al. 1996). However, the local levels used in our study were between 2000 and 3000 times lower than those toxic ones, and this dose corresponds to 1 g of vancomycin for 300 g of allograft, which corresponds to about 3 morselized femoral heads.

Our model was designed to permit the in vivo assessment of bone allograft incorporation with or without vancomycin in a weight-bearing limb devoid of fixation, which might affect healing. The tibia constitutes a large weight-bearing bone that can accept an oblong bone defect, which reduces the stress riser effect, and permits early function, despite the absence of fixation (Clark et al. 1977). We used allografts instead of autografts to resemble an acetabular or femoral impaction graft-

ing reconstruction, with a 90-day period for graft evaluation, which provided adequate time for the stimulation of osteogenesis.

Although high concentrations of vancomycin can substantially reduce osteoblast replication and even cause cell death (Miclau et al. 1993, Edin et al. 1996), the view that this antibiotic reduces bone graft incorporation was not supported by our findings. We found that high levels of vancomycin—i.e., up to 220 times the MIC—do not significantly affect bone graft incorporation.

The use of vancomycin-supplemented bone allografts may be of value in procedures performed at the site of a previously infected arthroplasty which require a second stage reconstruction with impacted bone grafting techniques.

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No competing interests declared.

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