In vitro release of vancomycin and tobramycin from impregnated human and bovine bone grafts

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In order to combine the effects of bone repair and eradication of infection, with both Gram-positive and Gram-negative pathogens, the behaviour of a compound of bone graft and antibiotics was investigated. Samples of human and bovine bone, cancellous and cortical, were processed and incubated with vancomycin and tobramycin, respectively. The compound was placed in 5% human albumin and the surrounding liquid was exchanged completely every 24 h. Concentrations of antibiotics in the fluid were measured over \( \leq 28 \) days using high pressure liquid chromatography and a bioassay. All tested combinations eluted mainly in the initial phase with a logarithmic decrease over the testing period. The concentration of antibiotics in the albumin was well above the MIC for common pathogens throughout the investigation in all tested specimens. The highest initial concentrations were measured in the compound of bovine bone together with vancomycin (24395.8 ± 1138.9 mg/L), decreasing to 9.02 ± 1.3 mg/L after 11 exchanges. Human and bovine bone did not have significantly different properties. The storage capability of cortical bone was generally lower than that of cancellous bone. Tobramycin concentrations were significantly lower in the initial phase; however, it eluted more steadily and over a longer period, so that from day 6 onwards, its concentration was greater than that of vancomycin. After 28 days, the tobramycin concentration was 18.09 ± 2.46 mg/L (bovine cancellous bone). In conclusion, bone, if processed adequately, is an excellent carrier for vancomycin and tobramycin. Cortical bone is as suitable as cancellous bone. The pharmacokinetics of human and bovine bone are comparable. Using an antibiotic–graft compound, eradication of pathogens and grafting of bony defects may be accomplished in a one-stage procedure.

Introduction

Management of bone infection and resulting bony defects is one of the major issues in orthopaedic surgery. Systemic antibiotic therapy alone does not usually eradicate bacteria because of poor penetration into bone. High doses and/or lengthy treatment with antibiotics are often not possible because of adverse effects.\textsuperscript{1–4} Additional problems arise with the increased prevalence of highly resistant pathogens such as methicillin-resistant \textit{Staphylococcus aureus} (MRSA).\textsuperscript{5} Chronic cases can only be treated by complete removal of all non-viable tissue followed by systemic and local treatment of the infection site.\textsuperscript{6–9} Local application of antibiotics can provide high drug concentrations at the site of infection and can avoid systemic effects.\textsuperscript{10,11} Impregnated implants have been developed; the most widely evaluated of these is poly-methyl methacrylate (PMMA) in combination with gentamicin.\textsuperscript{12–18} These implants have several disadvantages, however: (i) non-resorbable implants have to be removed, leaving empty spaces, which have to be filled, usually requiring further surgery; (ii) gentamicin is not the agent of choice for infections with Gram-positive strains. (iii) although concentrations of locally applied antimicrobial agents initially exceed those achievable by systemic application, these high concentrations are short-lived. Once

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To overcome these problems, an antibiotic carrier is needed which should provide: (i) effective bactericidal activity against all causative pathogens, including MRSA; (ii) a sustained, high concentration at the site of infection without local or systemic toxicity; and (iii) repair and healing of bone defects without further surgery.

Vancomycin is effective against most Gram-positive pathogens and is the agent of choice for infections with MRSA. Staphylococci, both coagulase-positive and -negative, are susceptible to vancomycin at concentrations of \( \geq 1-5 \) mg/L, making this ideal for treating infection with Gram-positive strains. Tobramycin is effective against many Gram-negative and Gram-positive pathogens, covering the majority of the relevant spectrum in orthopaedic surgery. Combining vancomycin and/or tobramycin with a resorbable or, even better, osteoconductive carrier, can provide effective antibiotic concentration at the site of infection and avoid the need to remove the carrier.

Polylactates or collagen sponges are used clinically as resorbable carriers, but they do not restore bone stock and are not capable of mechanical loading. Allogeneic cancellous bone has been proven to be effective in restoration of bone stock. Vancomycin- or tobramycin-impregnated bone grafts have been used clinically for filling infected bone defects after osteomyelitis and in exchange procedures after replacement of infected joints. However, a standardized incubation technique has not yet been established and the concentrations of antibiotics reached inside the graft and at the site of infection have not yet been investigated.

We have developed a preparation technique that gives reproducibly high quantities of vancomycin and tobramycin inside bone grafts. The aim of this study was to measure the kinetics of elution of vancomycin and tobramycin from impregnated bone of human and bovine origin.

**Materials and methods**

**Selection and preparation of bone grafts**

Bone of human origin was obtained from organ donors according to national and international regulations. We only used bone that was unsuitable for transplantation because of incomplete serological testing. Bone was harvested from the femur and the proximal tibia, and was separated into cancellous and cortical parts. After removal of adhering soft tissue it was cut into pieces of 1–5 mm side length. Fatty bone marrow, cells and small amounts of remaining soft tissue were removed by means of a series of shaking baths in ether, 70%, 50% and 30% ethanol and hydrogen peroxide. The bone was then frozen at \(-50^\circ \text{C}\) and freeze-dried to a residual moisture of \(<5\%\) water content. The dry bone was sealed in bottles containing argon and was irradiated using a \( ^{60}\text{Co} \) source with a dosage of 25–30 kGy at room temperature. The specimens were stored at room temperature until incubation.

In a second series, commercially available bone of bovine origin (Lubboc; Ost-Developpement, Clermont-Ferrand, France) registered for clinical transplantation was evaluated. The patented manufacturing process eliminates tissue and cellular elements contained in intr trabecular spaces, but leaves type 1 collagen fibres of the cancellous bone intact. Granules with an average diameter of 3.5 mm and two cubes (5 mm sides) were freeze-dried and irradiated as described above.

Vancomycin and tobramycin were obtained from Eli Lilly, Indianapolis, IN, USA. They were dissolved in distilled water, vancomycin at 1 g/10 mL and tobramycin at 800 mg/10 mL. Grafts were incubated in these solutions for 24 h, then rinsed twice in saline. The remaining solution served as a control.

One vancomycin-impregnated human spongy compound sample was freeze-dried again and stored at room temperature for 3 weeks. Evaluation of this specimen started 24 h after rehydration in water.

Six samples, weighing 1 g, were chosen from the granulate specimens, and two blocks of bovine vancomycin-incubated bone were placed in 3 mL of 5% human albumin at \(37^\circ \text{C}\). The albumin was replaced completely every 10 days, and thereafter on days 13, 15, 20, 22 and 28. All samples collected during the study were stored in liquid nitrogen until assayed for antibiotic content.

**Measurement of antibiotics**

Vancomycin concentrations were measured using high pressure liquid chromatography (HPLC), and tobramycin concentrations using bioassay. The chromatographic system consisted of a Shimadzu S/L6B autoinjection port, a Shimadzu LC9A pump and a UV–visible 240 nm SPD-10AV Shimadzu LC workstation (Shimadzu, Tokyo, Japan), with a CO1 UDHSE column. The mobile phase consisted of 5–25 mL of 50 mM KH\(_2\)PO\(_4\) and 10–20% (w/w) acetonitrile buffered with H\(_3\)PO\(_4\) to a pH of 2.5–3. The flow rate was 1 mL/min. The effluent was monitored at 240 nm. A solution of 10 mg/\(\beta\)-hydroxypropyltheophylline in 10 mL of HPLC-grade water served as an internal standard. One millilitre of this stock solution was diluted with acetonitrile to give a total volume of 10 mL, resulting in an internal standard concentration of 100 mg/L. The extraction procedure and chromatographic conditions were as follows: 1 mL of albumin sample was added to 2 mL of acetonitrile (pH 2.5–3), vortexed for 60 s and centrifuged at 7500g for 150 s. Tissue samples were weighed and diluted with 0.9% saline, so that 1 mL contained 100 mg of tissue. The samples were then homogenized, vortexed in 1.5–2 mL 50 mM KH\(_2\)PO\(_4\) for approximately 60 s and centrifuged at 14000g for 10 min. Twenty microlitres of the supernatant were injected into the HPLC column through the autoinjector. The lower detection limit was 0.45 mg/L.
Antibiotic-loaded bone grafts

Tobramycin concentrations in the supernatant were measured by means of a microagar diffusion test with antibiotic agar 1 (Merck, Darmstadt, Germany) and S. aureus ATCC 65389 as the test organism, used at a final concentration of approximately $10^6$ cfu/mL on the assay plate. Standards were placed into wells of the assay plates at concentrations of 160 to 0.156 mg/L (log2 dilutions). Assay plates with standard and sample were incubated overnight at 37°C and zones of inhibition were read to the nearest 0.1 mm. The lower detection limit of the assay was <0.156 mg/L.

Statistical analysis

The TESTIMATE software package (Test & Estimation; IDV, Gauting) was used for statistical calculations. $P$ values of $<0.05$ were considered statistically significant. The Wilcoxon–Mann–Whitney U-test was used for comparisons between the different groups. The release of active agent on each day and the area under the curve (AUC) was compared from day 1 to day 28. The significance level (i.e. the probability of rejecting $H_0$ when it is true) was 0.05; the type II error was 0.1.

Results

The elution properties of all tested combinations were similar, though certain differences could be found:

Vancomycin (Figure 1)

Human cancellous bone. Human cancellous (spongy) bone in combination with vancomycin generated initial mean vancomycin concentrations of 20904.66 ± 1844 mg/L (range, 18699.58–23443.73 mg/L), decreasing to 4.43 ± 0.95 (3.27–5.95) mg/L after 11 complete exchanges.

Human cortical bone. Vancomycin-impregnated cortical bone behaved significantly differently from cancellous bone (AUC, $P = 0.0022$). However, this difference was limited to the initial phase with mean day 1 concentrations of 5752.16 ± 2167 mg/L. The decrease was markedly slower than with cancellous bone. From day 9 to 13 there was no statistically significant difference between cancellous and cortical bone, and by day 13 the concentrations were comparable (5.15 ± 1.61 mg/L).

Bovine cancellous bone. Vancomycin concentrations were higher with bovine cancellous bone than with human bone. After 13 days, the mean concentrations were twice those in human bone, but the overall difference was not statistically significant ($P = 0.5887$). There was no statistically significant difference between the behaviour of 5 mm cubes of bovine cancellous bone and that of morselized samples ($P = 0.2857$).

Effect of lyophilization. Lyophilization and rehydration of morselized human cancellous bone did not have a significant effect on elution ($P = 0.0649$).

Tobramycin (Figure 2)

Cancellous human bone. Cancellous human bone yielded significantly lower concentrations of tobramycin than of vancomycin, especially in the first phase (until day 9) ($P = 0.0022$). The decrease in concentration, however, was markedly slower for tobramycin. After 13 days the concen-
Concentrations were similar to those of vancomycin, and after 28 days (15 exchanges) the concentrations were still well above the MIC.

Cortical human bone. With cortical bone, initial tobramycin release was about half that with cancellous bone; the decrease in concentration was equal in both types of bone. After 22 days tobramycin concentrations in the cortical bone samples were below the MIC.

Bovine cancellous bone. Initial concentrations were markedly higher than with human bone and decreased much more slowly. The difference between bovine and human bone was statistically significant on each day ($P < 0.0022$). After 28 days, concentrations were still therapeutic (18.09 mg/L). There was no statistically significant difference between structural and morselized bovine grafts in the elution properties of tobramycin ($P = 0.0714$).

Discussion

The use of bone grafts as a carrier for antibiotics has been described by few authors. McLaren & Miniaci mixed vancomycin and tobramycin with freshly harvested cancellous bone; after clinical implantation, concentrations in the elution fluid were two to 10 times the systemic toxic concentrations. These findings were confirmed by Hernigou et al., who, using the same technique, found concentrations of between three and 15 times the systemic toxic concentration. Changing the fluid every day in vitro, vancomycin concentrations of 625 mg/L were found on the first day, decreasing to 1.7 mg/L on day 15. The main disadvantage of the reported method is the use of autologous bone, which is not always readily available.

It is believed that cleaning the bone to remove fat, bone marrow and soft tissue makes it easier for the solution to penetrate through the canaliculi of the cancellous structures, increasing the surface available for adsorption. Gunal et al. investigated the release of gentamicin, ciprofloxacin and penicillin G, from deproteinized xenograft of bovine origin into saline. Eight hours of incubation were found to be sufficient for maximum saturation of the carrier. Concentrations were 5 mg/L after 24 h, decreasing to 0.5 mg/L after 10 days. The authors thought that these concentrations would be sufficient to eradicate bacteria, although their results indicated lower concentrations than those reported by McLaren and Hernigou et al.

In the present study a compound of a carrier and antibiotics was created with the aim of increasing concentrations inside the carrier, to provide high initial concentrations in the surrounding liquid and prolonged release in the following weeks and months. At the same time, the carrier should ideally provide mechanical support and should repair defects. We developed a method consisting of several steps, each of which increases the loading capability. Drying the carrier was considered important for success, as tissue in a completely dry state is likely to absorb much higher quantities of aqueous solution during rehydration than a non-dehydrated tissue. Consequently, a dry tissue will bind water together with agents solubilized therein much more strongly. Of course, a certain amount of hydratable substance is required to provide the desired effect. In the samples described here, the hydratable substance was

![Figure 2. Elution of tobramycin from fresh human cancellous bone (■); fresh human cortical bone (●); and fresh cancellous bovine bone, either morselized (■) or in a block (●).](image-url)
collagen, embedded in a crystalline scaffolding. It is crucial, therefore, to maintain the amount of natural collagen inside the graft during preparation; this was achieved by lyophilization. All other processing steps were designed to increase further the accessibility of the solution to the carrier as well as binding to it. The presented method increases storage capability by about 100-fold and results in closer binding of substances to the carrier, prolonging the release of vancomycin and tobramycin into the surrounding tissue and increasing the concentrations achieved. In general, vancomycin was released more quickly than tobramycin.

For clinical use in cases of chronic infection of bone, efficacy of antibiotic release is of major importance. Lack of local toxicity is also important, and mechanical properties must be suitable. Edin et al. showed that vancomycin 1000 mg/L did not adversely affect osteoblast replication. The same group found that tobramycin 400 mg/L significantly decreased cell replication but there were no detectable differences in the healing characteristics of cancellous bone graft with or without tobramycin as seen on X-rays, microradiographs, bone density analyses, histological examination and biomechanical testing. Both antibiotics are more compatible with osteoblast replication than others, such as cefazolin or ciprofloxacin. Whether the mechanical properties of lyophilized bone are impaired by antibiotic impregnation requires further evaluation, although this might be of minor importance as long as the grafts are not load-bearing.

This study has shown that large amounts of vancomycin and tobramycin can be bound inside bone, provided that the processing method is adequate. The resulting bone–antibiotic complexes release antibiotics into the surrounding medium, reaching high peak concentrations initially and then decreasing and becoming steadier for longer periods. This seems to fulfill the conditions needed to treat bone infection, where initial concentrations need to be high over several days the concentrations can be reduced; the continued presence of the antibiotic should prevent recontamination of the graft and allow antibiotic to reach more distant parts of the operative site. Thus, neo-osteogenesis, which starts only several days after grafting, is not likely to be compromised. Both human and bovine bone performed very well as carriers, though minor differences in binding capacity were seen. Cortical bone was less accessible to antibiotics than cancellous bone, resulting in lower initial concentrations of antibiotic. Long-term elution from the two types of bone, however, was comparable. Because of its smaller surface area, cortical bone may not be able to bind so much antibiotic; in contrast, the amount of molecules directly bound to collagen seems to remain more or less equal.

In conclusion, our results suggest that vancomycin or tobramycin in combination with adequately prepared bone graft carriers are well suited for the effective local treatment of bone infection.

References


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