Release of netilmicin and vancomycin from cancellous bone

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Submitted 01-02-22. Accepted 01-08-30

ABSTRACT – First, we studied the effect of the following variables used for netilmicin- and vancomycin-impregnation of cancellous bone: a) antibiotic concentration of the impregnation fluid, b) time used for impregnation, c) pH of the impregnation fluid, d) the degree of bone morselizing and e) antibiotic combination. An increase in the antibiotic concentration of the impregnation fluid increased the amount of antibiotics released from bone. In addition, the amount of vancomycin eluted was also dependent on the time used for impregnation. The fraction of the total amount of netilmicin and vancomycin released after 24 h was 80% and 30%, respectively. More netilmicin and vancomycin were eluted from bone impregnated with antibiotics at pH 7 than the amount eluted from bone impregnated at pH 3. More netilmicin was eluted from fine morselized bone than from coarse morselized bone. By combining netilmicin and vancomycin in the impregnation fluid, the release of vancomycin was reduced. Secondly, we analyzed if the release of antibiotics from bone was complete: 99.9% of the total amount of netilmicin adsorbed to the bone was released by elution during 6 weeks. Finally, after implantation of netilmicin-impregnated bone in rabbit femur condyle, we measured netilmicin and vancomycin in serum: peak serum values of netilmicin were 4.2 (3.7–4.7) mg/L 2–3 h postoperatively.

Local delivery systems for antibiotics have been studied in bone cement, polymethylmethacrylate beads and biodegradable carriers (Wahlig and Dingeldein 1980, Buchholz et al. 1981, Walenkamp 1983, Frauhofer et al. 1985, Wroblewski 1986, Calhoun and Mader 1989, Ipsen et al. 1991, Adams et al. 1992, Shinto et al. 1992, Becker et al. 1994, Nie et al. 1995, Mader et al. 1997, Masri et al. 1998). Numerous studies have been done on cancellous bone as an antibiotic carrier (De Grood 1947, Petri 1984, Hernigou et al. 1991, Miclau et al. 1993, Chan et al. 1998, 2000, Witsø et al. 1999, 2000, Winkler et al. 2000). However, a review of the literature showed no reports on variables that may affect the adsorption and subsequent release of antibiotics from cancellous bone.

We therefore studied the amount of netilmicin and vancomycin released as a function of the following variables used for impregnation of morselized cancellous bone: a) antibiotic concentration of the impregnation fluid, b) time used for impregnation, c) pH of the impregnation fluid, d) the degree of bone morselizing and e) the amount of antibiotics released when combining netilmicin and vancomycin in the impregnation fluid. We also studied if in vitro release of netilmicin from the antibiotic-impregnated bone was complete. Finally, we determined to what extent implantation of netilmicin- or vancomycin-impregnated cancellous bone results in detectable serum levels in a rabbit model.

Material and methods

We used a modification of the technique for adsorption and subsequent release of antibiotics from morselized cancellous bone described in a previous study (Witsø et al. 1999). Human cancellous bone was passed through a bone mill (Aesculap, HARRIS, GB 043 and GB 044) under sterile conditions to yield fine and coarse morselized cancellous bone, respectively. Netilmicin sulfate powder

and vancomycin powder were dissolved in sterile water. The pH of the different antibiotic solutions was adjusted by adding either 6 M HCl or 10 M NaOH. 10 g of morselized cancellous bone was placed in a small polypropylene tube with 10 mL of antibiotic solutions at room temperature. The fluid was cleared from the bone by straining it through gauze. When the gauze was twisted, it left almost dry bone. The antibiotic-impregnated bone was compressed into wire-mesh cylinders in triplicate. Each cylinder contained 1.00 (0.97–1.02) g of antibiotic-impregnated bone. The cylinder with its contents was placed in an elution tube (15 × 100 mm sterile glass tube) with 5 mL of phosphate-buffered saline (PBS, pH 7.2) at 37 °C for 24 h. After a standard washing procedure in 5 mL of saline, the cylinder was transferred into a new elution tube. The study continued with daily transfer of the cylinder. Each day PBS from the elution tube was frozen in aliquots at –70 °C and stored pending analysis of the antibiotic concentration. Bone impregnated with saline was used as a control.

Antibiotic concentration assessed by immunoassay

We determined netilmicin and vancomycin concentrations using fluorescence polarization immunoassay technology (TDx/TDxFLx vancomycin and netilmicin Assay System). In this assay, 2.0 mg/L of vancomycin and 0.09 mg/L of netilmicin are the lower detection limits.

Physiochemical factors and influence on antibiotic adsorption and release

Time and concentration. Coarse morselized cancellous bone was impregnated in antibiotic solutions for 10 min, 60 min and 24 h. Two antibiotic concentrations were used. The pH of the different antibiotic solutions was adjusted to pH 5.3 ± 0.1. When the concentration of netilmicin in the elution fluid remained ≤ 1 mg/L for 7 days, the antibiotic-impregnated bone was eluted weekly in 35 mL PBS until the netilmicin concentration was < 0.09 mg/L. Vancomycin was eluted from the bone until two consecutive vancomycin concentrations were < 2.0 mg/L.

pH. Coarse morselized cancellous bone was impregnated in netilmicin (100 mg/mL) and vancomycin (100 mg/mL) solutions at pH 3, 5 and 7 for 10 min. The elution was continued for 14 days.

Degree of bone morselizing. Fine and coarse morselized cancellous bone was impregnated in netilmicin solution, 400 mg/mL, pH 5.2, and vancomycin solution, 100 mg/mL, pH 5.2 for 10 min. The elution was performed during a period of 14 days.

Combination of netilmicin and vancomycin. Coarse morselized cancellous bone was impregnated for 10 minutes in three different antibiotic solutions at pH 5.3: a) netilmicin, 100 mg/mL, b) vancomycin, 100 mg/mL and c) vancomycin 100 mg/mL + netilmicin, 100mg/mL. The elution was continued for 14 days.

Extraction of potential residual amounts of antibiotics. We used a modification of methods described in previous reports (Gerhardt et al. 1994, Carlson et al. 1997). Briefly, after 6 netilmicin-impregnated bone cylinders had been eluted to netilmicin levels < 0.09 mg/L, the bone was dissolved in 5 mL of 7% perchloric acid for 60 min in a test tube. After shaking for 60 seconds and centrifugation (3000 × g for 10 minutes), the netilmicin concentration was assessed in the supernatant. Preliminary studies had shown that processing a netilmicin solution for 60 minutes with perchloric acid did not affect the netimicin concentration assessed by immunoassay.

In vivo studies

Wire mesh cylinders were filled with fine morselized cancellous rabbit allograft impregnated for 10 min with netilmicin 400 mg/mL or vancomycin 100 mg/mL. Each cylinder contained 0.58 (0.54–0.62) g of bone. 6 New Zealand outbred, female rabbits, 4.3 (4.1–4.6) kg were operated on under sterile conditions. A transcondylar femoral canal was made with an eight mm drill. The cylinder filled with antibiotic-impregnated bone was implanted in the transcondylar canal (Figure 1). Each animal received one cylinder and the study was performed in triplicate.

Serum samples for analyses of antibiotics were taken preoperatively and 1, 2, 3, 4, 24 and 48 hours after implantation. The peroperative anesthesia consisted of 1.6–1.8 mL midazolam 5 mg/mL and 1.2–1.6 mL fentanyl-fluanisone intramuscularly. As postoperative sedation, each animal received a subcutaneous injection of 0.5 mL buprenorphinum after 5h and 18h. After 48 h the rabbits were killed

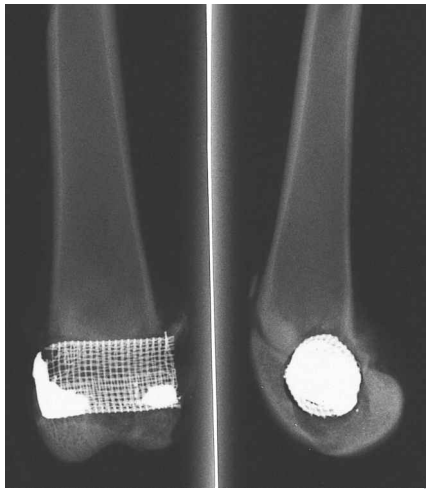


Figure 1. Rabbit morselized cancellous allograft was impacted into wire-mesh cylinders with solid bottom (diameter 8 mm, height 14 mm). Pilot studies had shown that the transcondylar diameter of New Zealand rabbits was about 15 mm. After predrilling, the cylinder was inserted in the femoral condyle under sterile conditions from the lateral side, so that the cylinder lay flush with the corticalis on the medial side. On the lateral side, the cylinder was sealed with bone wax.

with an intravenous injection of 3 mL pentobarbital (100 mg/mL).

The study was approved by the Norwegian Council for Animal Experimentation.

Statistics

If not otherwise stated the results are presented as arithmetic mean (dispersion as total range).

Analysis of variance was performed using ANOVA. If a statistically significant difference was detected, a post hoc test was used to test pairs of means (Fisher's LSD multiple-comparison test, Duncan's multiple-comparison test. NCSS 2000, Statistical System for Windows. Number Cruncher Statistical Systems, Kaysville, UT, USA).

Statistical significance was considered at $p \leq 0.05$.

Results

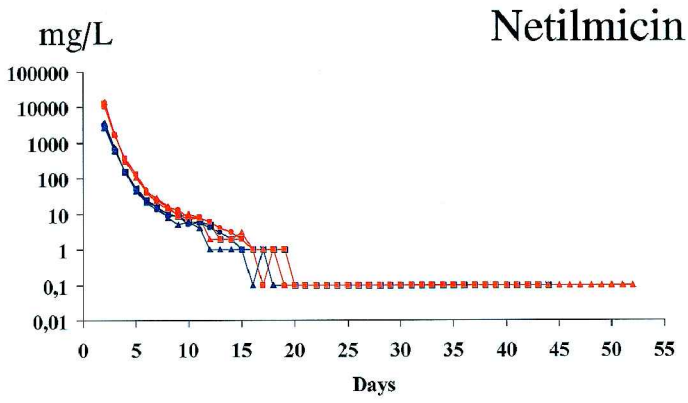
The effect of different antibiotic concentrations of impregnation fluid and time used for impregnation. Neither netilmicin nor vancomycin was eluted from control bone.

A fourfold increase in the netilmicin concentration of the impregnation fluid caused a 252 (200–316)% increase in the amount of netilmicin subsequently released from the bone. The time used for impregnation had no significant effect on the amount of netilmicin subsequently released (Table). The elution profile of netilmicin from netilmicin-impregnated bone showed an exponential decay (Figure 2). After 24 hours, about 80% of the total amount of netilmicin was released (Table). The total elution time was 42 (26–55) days.

Both the vancomycin concentration of the impregnation fluid and the time used for impregnation influenced the subsequent release of vancomycin. Peak values and the total amount of vancomycin eluted from bone impregnated in 100 mg/mL vancomycin solution for 24 h were higher than the amount of vancomycin eluted from the other 5 groups. By increasing the vancomycin concentration of the impregnation fluid fourfold, the amount of vancomycin released increased by 125 (69–155)%, irrespective of the time used for impregnation (Table). The amount of vancomycin eluted also showed an exponential decay throughout the study period (Figure 2). However, only about 30% of the total amount was eluted during the first 24 hours (Table). The total elution time was 32 (26–36) days.

Influence of pH. The total amounts of netilmicin eluted during 14 days from bone impregnated at pH 3, pH 5 and pH 7 were 14 (13–15) mg, 19 (18–20) mg and 21 (19–22) mg, respectively ($p = 0.001$). Bone impregnated with netilmicin at pH 3 eluted less antibiotics than bone impregnated at pH 5 or pH 7. The total amounts of vancomycin eluted during 14 days from bone impregnated at pH 3, pH 5 and pH 7 were 31 (25–42) mg, 38 (29–45) mg and 57 (55–60) mg, respectively ($p = 0.01$). Bone impregnated with vancomycin at pH 3 or pH 5 eluted significantly lower amounts of antibiotics than bone impregnated at pH 7.

Degree of bone morselizing. The amount of netilmicin eluted from fine morselized bone was higher than the amount eluted from coarse morselized bone: 63 (56–67) mg vs 48 (46–51) mg, ($p = 0.02$). The amount of vancomycin eluted was not influenced by the degree of bone morselizing: 56 (53–60) mg vs 55 (44–63) mg.



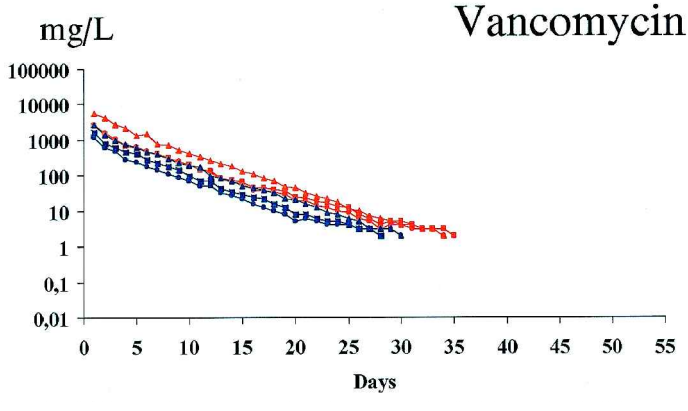


Figure 2. Netilmicin and vancomycin were eluted from antibiotic-impregnated morselized cancellous bone in vitro. The pattern of release was assessed as a function of the antibiotic-concentration in the impregnation fluid (C1 = low concentration, C2 = high concentration) and the time used for impregnation (T1 = 10 minutes, T2 = 60 minutes, T3 = 24 h). Only mean values are listed in the figure. C1T1: ●, C2T1: ●, C1T2: ■, C2T2: ■, K1T3: ▲, K2T3: ▲.

Release of netilmicin and vancomycin from cancellous bone

Groups studied	Total amount mg	Peak values mg/L	% of total amount eluted during		
			24 h	72 h	120 h
Netilmicin					
100 mg/mL–10 min	21 (16–27)	3153 (2330–4360)	76 (73–82)	97 (95–98)	99 (98–99)
400 mg/mL–10 min	63 (51–87)	10477 (8260–14880)	81 (79–85)	98 (97–98)	99 (99–99)
100 mg/mL–60 min	18 (16–21)	2753 (2430–3200)	75 (74–76)	96 (96–96)	98 (98–98)
400 mg/mL–60 min	75 (59–84)	12717 (9590–14670)	85 (82–88)	98 (98–98)	99 (99–100)
100 mg/mL–24 h	24 (17–28)	3820 (2770–4380)	79 (79–80)	98 (97–98)	99 (99–99)
400 mg/mL–24 h	82 (67–98)	13970 (11230–17220)	85 (83–88)	98 (98–99)	99 (99–100)
Vancomycin					
25 mg/mL–10 min	18 (15–20)	1143 (958–1314)	32 (28–36)	63 (60–65)	77 (75–79)
100 mg/mL–10 min	45 (41–47)	2684 (2513–2782)	30 (29–31)	59 (57–60)	74 (72–76)
25 mg/mL–60 min	26 (23–27)	1599 (1400–1720)	31 (31–32)	59 (54–62)	76 (70–80)
100 mg/mL–60 min	44 (32–50)	2585 (1901–3082)	30 (28–31)	58 (55–60)	72 (70–74)
25 mg/mL–24 h	42 (42–45)	2625 (2550–2685)	31 (30–33)	58 (56–60)	74 (72–76)
100 mg/mL–24 h	107 (98–122)	5611 (4623–6234)	26 (23–30)	59(56–64)	75 (72–80)

The amount of antibiotic eluted from antibiotic–impregnated cancellous bone was assessed as a function of the antibiotic concentration of the impregnation fluid and time used for impregnation. The total amount of antibiotic eluted was calculated as the summation of the amount eluted each day in the study period. Peak values were the maximum concentration measured in the elution fluid (during the first 24 hours for all groups).

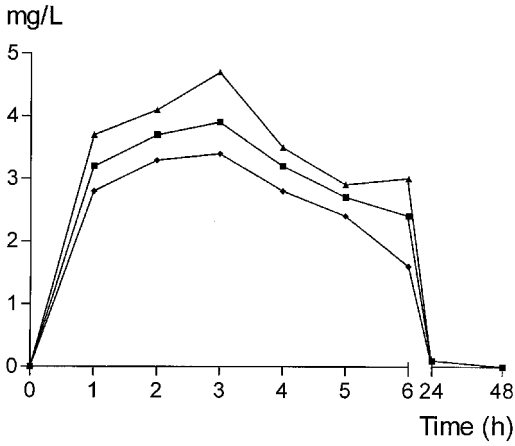


Figure 3. Netilmicin- and vancomycin-impregnated bone were implanted in the rabbit femoral condyle in vivo. Only netilmicin was detectable in serum postoperatively.

Effect of combining netilmicin and vancomycin. The amount of netilmicin eluted from bone impregnated with netilmicin + vancomycin and netilmicin solutions were similar: 19 (18–19) vs 18 (17–19) mg. However, the amount of vancomycin released from bone impregnated in the combined antibiotic solution was reduced compared to the amount of vancomycin released from bone impregnated with vancomycin alone: 30 (29–32) mg vs 40 (35–45) mg ($p = 0.03$).

Extraction of potential residual amounts of netilmicin. After processing with perchloric acid, 18 (4–30) μg of netilmicin was released from bone previously eluted in PBS until the netilmicin concentration in PBS did not differ significantly from zero. This residual amount of netilmicin was about 0.1% of the total amount eluted from the bone during 42 days.

In vivo studies. Netilmicin and vancomycin were not detectable in serum preoperatively. In animals operated on with implantation of netilmicin-impregnated bone, the netilmicin peak serum values were 4.2 (3.7–4.7) mg/L 2–3 h postoperatively (Figure 3). Vancomycin could not be detected in serum postoperatively.

Discussion

By optimizing the conditions for antibiotic impregnation of bone, more than 70 mg of netilmicin and more than 100 mg of vancomycin were released from 1 g of cancellous bone in vitro.

We used a standardized technique for elution of antibiotics from bone. When studying elution of antibiotics from bone cement and biodegradable materials, PBS, serum, sterile water and Mueller Hinton broth have been used as the elution fluid (Picknell et al.1977, Baker and Greenham 1988, Becker et al. 1994, Adams et al.1992, Miclau et al. 1993, Mader et al. 1997, Klekamp et al. 1999, Witsø et al. 1999, 2000). When comparing the elution of netilmicin and vancomycin into broth or PBS, peak values and elution profiles are similar (Witsø et al. 1999). Concerning the amount of antibiotic eluted, a considerable disperse between the three parallels was observed. Morselized bone was probably not as homogeneous as expected.

Almost all netilmicin adsorbed into bone was eluted in PBS during 6 weeks. By contrast, antibiotic is eluted from bone cement for many years in vitro and less than 20% of the total amount of antibiotic mixed in bone cement is released (Picknell et al. 1977, Wahlig and Dingeldein 1980, Miclau et al. 1993).

The pH of the impregnation fluid affected the amount of antibiotics released from cancellous bone. Like all polar molecules, netilmicin and vancomycin can form hydrogen bonds with other polar molecules. The polarity of the antibiotics and the bone is probably strongly influenced by pH. Almost twice as much netilmicin and vancomycin was released from cancellous bone impregnated at pH 7 than from bone impregnated at pH 3. In clinical practice, vancomycin powder has been mixed with cancellous bone autograft (Chan et al. 1998, 2000). A vancomycin solution is acidic (Inman and Winely 1994). A mixture of bone and vancomycin powder would probably also be acidic, resulting in suboptimal conditions for antibiotic impregnation of the bone.

We found that in vitro elution of netilmicin and vancomycin from antibiotic-impregnated morselized cancellous bone was intense early with an exponential decay, similar to previous studies (Witsø et al. 1999, 2000, Winkler et al. 2000). The in vitro elution of netilmicin showed a more rapid decay than vancomycin. An in vitro study showed a slower release of tobramycin from cancellous bone than of vancomycin (Winkler et al. 2000).

This apparent difference between two aminoglycosides compared to vancomycin may result from different elution properties or different methods used for impregnation of antibiotics—e.g., adjustment of pH. Unlike studies on the release of aminoglycosides and glycopeptides from bone cement and PMMA beads, peak values and the total amount of vancomycin eluted from cancellous bone in vitro were not less than those of netilmicin (Adams et al. 1992, Mader et al. 1997, Klekamp et al. 1999).

When considering the in vitro release of netilmicin and vancomycin from cancellous bone, we found rather unexpected differences between the two antibiotics. First, only the release of vancomycin was influenced by the time used for antibiotic impregnation of bone. Secondly, unlike to netilmicin, the amount of vancomycin released decreased when the two antibiotics were combined in the impregnation fluid. Finally, the release of vancomycin was not affected by increasing the surface area. In addition to the larger molecular mass of vancomycin, 1449 Da vs. 476 Da, quantitative and qualitative differences with respect to the nature of antibiotic binding sites on cancellous bone may explain these observations.

Experimental osteomyelitis in animals has been used to study antibiotic release from locally implanted materials (Gerhardt et al. 1993, Riegels-Nielsen et al. 1995). In our study, implantation of 0.6 g of netilmicin-impregnated cancellous bone in rabbits resulted in peak serum levels of 4.0 mg/L netilmicin. In total hip revisions usually 1–2 femoral heads (50–200 g) are morselized and used for bone impaction. According to our findings, 100 g of netilmicin-impregnated cancellous bone can elute more than 7000 mg of netilmicin in vitro, which may be toxic in humans. However, in the rabbits, the serum values would probably have been smaller if the cylinder had been placed in a sclerotic femur.

Our observations actualize the use of netilmicin-impregnated cancellous bone as a prophylactic option when doing revision hip and knee arthroplasties and using of cancellous bone impaction. However, our study calls for caution because impaction of large amounts of netilmicin-impregnated cancellous bone may cause toxic netilmicin values in serum.

We thank Schering-Plough A/S, Norway, for supplying us with netilmicin sulfate powder.

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