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Effect of pulsed ultrasound in combination with gentamicin on bacterial killing of biofilms on bone cements *in vivo*

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Abstract

Aim—The aim of this study is to investigate whether pulsed ultrasound in combination with gentamicin yields increased killing of bacterial biofilms on bone cements *in vivo*.

Methods and Results—Bacterial survival on bone cement in the presence and absence of ultrasound was compared in a rabbit model. Two bone cement samples with *E. coli* ATCC 10798 biofilm were implanted in a total of nine rabbits. In two groups bone cement disks loaded with gentamicin were used, and in one group unloaded bone cement disks in combination with systemically administered gentamicin were used. Pulsed ultrasound with a mean acoustic intensity of 167 mW cm⁻² and a maximum acoustic intensity of 500 mW cm⁻² was applied from 24 h till 72 h post surgery on one of the two implanted disks. After euthanization, the bacteria removed from the disk were quantified. Application of ultrasound, combined with gentamicin, reduced the biofilm in all three groups varying between 58 to 69% compared to the negative control. Ultrasound proved to be safe with respect to creating skin lesions.

Conclusions—Ultrasound resulted in a tendency of improved efficacy of gentamicin, either applied locally or systemically.

Significance and impact of Study—This study implies that ultrasound could improve the prevention of infection, especially because the biomaterials, gentamicin and ultrasound used in this model are all in clinical usage, but not yet combined in clinical practice.

Keywords

Biofilm; bone cement; antimicrobial; ultrasound; *in vivo* test

INTRODUCTION

Biomaterial related infections are difficult to eradicate because the biofilm mode of growth protects the infecting organisms against environmental attacks (Gristina and Costerton 1985). In the biofilm mode of growth, bacteria are embedded in an exopolymeric matrix, which contributes to effectively protecting the organisms against antimicrobial therapy, although the

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exact mechanisms of increased antibiotic resistance of bacteria in biofilms are not known. Current hypotheses include a slow or incomplete penetration of the antibiotic into the biofilm, an altered chemical microenvironment within the biofilm, or the formation of bacterial sub-populations in a resistant phenotypic state (Stewart and Costerton 2001).

Biomaterial related infections cause failure of total hip and knee arthroplasties in 1% to 5% of the almost half a million surgeries annually performed worldwide (Mariani and Tuan 1998; Bauer and Schils 1999). Acrylic bone cement is widely used to fixate these joint prostheses, and it is assumed that loading bone cement with an antibiotic may decrease the infection rate by local release of antibiotic (Persson *et al.* 1999; Walenkamp 2000). Advantages of these local drug releasing systems are that a high concentration of the drug can be achieved locally and possible side-effects of high systemic drug concentrations can be limited or avoided. Despite high initial release rates, antibiotic release rates over time are low, and about 85% of the antibiotic is retained in currently applied bone cements (Wroblewski *et al.* 1986; Kuechle *et al.* 1991; Powles *et al.* 1998). It was hypothesized that the antibiotic release pattern of antibiotic-loaded bone cement is partially a surface phenomenon whereas the total amount of antibiotic released depends on bulk porosity (Van de Belt *et al.* 2000a).

Ultrasound has been used in various medical applications, including medical diagnostic imaging and physiotherapeutic treatment (Ter Haar 1987). Recently, ultrasonically activated drug delivery in chemotherapy was tested *in vivo* (Nelson *et al.* 2002). More important for this study, ultrasound is also effective in enhancing the efficacy of antibiotics, defined as the :“bioacoustic effect” (Qian *et al.* 1994), whereas ultrasound itself proved not to influence bacterial viability in biofilm (Qian *et al.* 1994; Rediske *et al.* 1999). Exposure of an *Escherichia coli* biofilm on polyethylene to low-frequency ultrasound for 24 h in a 1:3 duty cycle with a peak acoustic intensity of 500 mW/cm² and a temporal mean acoustic intensity of 167 mW cm⁻² yielded enhanced killing of the organisms by gentamicin in rabbits (Rediske *et al.* (2000). Presumably, ultrasound induces cavitation within the biofilm, which increases transport of solutes through the biofilm or outer bacterial membranes (Carmen *et al.* 2004b, Carmen *et al.* 2004c).

Ideally, to prevent biofilm infection of implants, a high concentration of antibiotic should be achieved around an implant immediately after surgery to eradicate planktonic bacteria, present as an inevitable result of contamination during surgery. The bioacoustic effect could optimize infection prevention. For example, antibiotic-loaded bone cement has been modeled as consisting of an insoluble matrix, with pores and channels in the bulk through which gentamicin can elute through diffusion (Cobby *et al.* 1974; Baker and Greenham 1988; Kuechle *et al.* 1991), which elution could possibly be accelerated by ultrasound (Hendriks *et al.* 2003).

Therefore, the aim of this study is to investigate whether pulsed ultrasound in combination with gentamicin yields an increased killing of bacterial biofilms on unloaded and gentamicin-loaded acrylic bone cement. To this end, we compared the survival of bacteria in a biofilm on implanted bone cement disks with and without application of ultrasound in a rabbit model.

MATERIALS AND METHODS

Preparation of bone cement disks and biofilm formation

Palacos, a bone cement without gentamicin, and Palacos G, a bone cement containing 0.84 w/w% gentamicin base were used. These two commercially available acrylic bone cements (Schering-Plough, Maarssen, The Netherlands) were prepared by mixing the powdered methylmethacrylate with the liquid monomer in a bowl with a spatula. Manual mixing was done according to the manufacturer's instructions and resulted in liquid cement. The liquid cement was poured into a polytetrafluorethylene mould and immediately pressed between two

glass plates for 25 min. After hardening, the circular cement disks with a total surface area of 8.17 cm² were pulled out of the mould and stored under dark, sterile conditions at room temperature.

In order to mimic aged gentamicin-loaded bone cement, as after several years of implantation, Palacos G was also studied after the initial release of gentamicin. To this end, 20 samples of Palacos G were immersed in 1 l of phosphate-buffered saline (PBS: solution of 10 mM potassium phosphate and 150 mM NaCl with a pH of 7.0) in a low rate shaking system (Gyrotory® water bath shaker Model G 76, New Brunswick Scientific Co. Inc., U.S.A.) at room temperature for 14 days. Thus prepared samples, will be referred to as “post-elution Palacos G” (PePaG).

E. coli ATCC 10798 was used, as described before (Rediske *et al.* 1999; Rediske *et al.* 2000; Carmen *et al.* 2004c), for biofilm formation on polyethylene. Although this strain is not a typical causative organism for orthopaedic implant related infections, it has been employed in previous studies on the effect of ultrasound on antibiotic efficacy. Twenty-four h prior to surgery, 10 ml of an overnight culture, grown in tryptic soy broth (TSB) at 37°C, was centrifuged at 4800 rpm for 10 min (GS-15R; Beckman, Fullerton, Calif.). The supernatant was decanted and the pellet was resuspended in 10 ml sterile PBS. Twenty µl of this suspension was added to 30 ml of TSB in sterile glass petri dishes, each containing one sterile bone cement disk. Cement disks were left in the petri dishes for 24 h, while rotating at 100 rpm at 37°C and refreshing the TSB every 8 h. In order to successfully establish a biofilm on PaG cement with similar numbers of CFU's as on unloaded cement, TSB was refreshed twice during the 24 h growth period.

Animal model

Nine New Zealand white female rabbits were housed and maintained under the regulations of the Institutional Animal Care and Use Committee of Brigham Young University and the U.S. Department of Agriculture. After pre-anesthetization with a combination of xylazine (5 mg kg⁻¹) and ketamine HCl (35 mg kg⁻¹), injected intramuscularly, the rabbits were denuded around the implant sites by shaving and the application of Nair® depilatory cream. After the rabbits were anesthetized with isoflurane delivered via a facemask, the denuded skin was aseptically prepared and two incisions were made perpendicular to the vertebral column. The implant sites were located just beneath the cutaneous trunci parallel to the vertebral column. An infected bone cement disk was inserted and sutured to the underside of the skin through two holes in each tab of the disk. A second infected bone cement disk was placed on the contralateral side. Immediately after surgery, and every 24 h thereafter, Banamine (2 mg kg⁻¹; flunixin-megalumine, Schering-Plough, Kenilworth, N.J.) was injected subcutaneously to relieve any pain. Blood samples were sampled daily prior to any injections and, after dilution, plated onto nutrient agar (NA) using the membrane filtration technique and incubated for 48 h.

The animals were divided in three experimental groups. One group received systemic gentamicin through a daily subcutaneous injection (8 mg kg⁻¹, Gentocin; Schering-Plough, Kenilworth, N.J.) after implantation of unloaded bone cement (Palacos, Pa, *n* = 3). Two groups were examined in the absence of systemic gentamicin after implantation of gentamicin-loaded cement (PaG, *n* = 3) or post-elution Palacos G (PePaG, *n* = 3).

Ultrasound was applied from 24 h till 72 h post surgery on one of the two implanted disks. The other disk acted as a negative control. A function generator (Hewlett Packard 3312A) created a sinusoidal wave at 28.48 kHz pulsed in a 1:3 duty cycle that was amplified by a RF amplifier (model 240L; ENI, Rochester, N.Y.). A 10:1 voltage transformer (Edo Acoustics, Salt Lake City, UT) was used to boost the voltage going to the ultrasound transducer (EDO Acoustics,

Salt Lake City, UT). To calibrate an acoustic intensity of 500 mW cm^{-2} during the pulse, the output signal was measured in advance by a calibrated hydrophone (Bruel and Kjaer, Naerum, Denmark).

The transducer was fixed on a rabbit using an acoustically conductive gel adhesive (Tensive; Parker Laboratories) inside a silicone rubber flange that was held in place with Kamar® adhesive (Kamar Inc., Steamboat Springs, CO) and a canvas jacket. An air stream of about 62 ml min^{-1} inside the flange transferred heat away from the transducer.

48 h after initiation of the ultrasound treatment, the rabbits were euthanized with a 1 ml intravenous injection of a solution composed of 26% sodium pentobarbital and 7.8% isopropyl alcohol (Sleepaway, Ft. Dodge, Iowa). Thereafter the implanted disks with biofilm were removed from the subcutaneous fascia and fibrous capsule. The tabs were cut from the disks and each circular disk (8.17 cm^2) was placed in a separate test tube, filled with 10 mL of 0.05% trypsin solution. A skin sample of the location of the transducer was removed from each rabbit and the kidneys from one rabbit in each experimental group were removed for histopathological examination.

Biofilm evaluation

For biofilm evaluation, the bone cement disks were sonicated in the test tubes in a Sonicator SC-100 sonicating bath (Sonicator Instrument Co., Copiaque, N.Y.) at 70 kHz for 10 s at 37°C to remove adherent bacteria; this was previously shown not to decrease bacterial viability (Carmen *et al.* 2004b). Bacteria removed from the disk were quantified by serial dilution in sterile physiological saline solution and membrane filtration using $0.45 \mu\text{m}$ cellulose acetate filters (Gelman Sciences, Ann Arbor, Mich.). The first tube in each dilution series contained 0.1% polysorbate 80 (Tween 80) to reduce bacterial clumping. After the filtration, the membranes were placed on NA plates and incubated at 37°C for 48 h. Colony forming units (CFU) were counted and CFU cm^{-2} on each disk were computed. Enhancement in bacterial killing was defined as the decrease in mean bacterial counts when ultrasound was applied.

RESULTS

The cement disks contained on average in all groups $2.3 \times 10^9 \text{ CFU cm}^{-2}$ of biofilm bacteria before implantation. After 72h of implantation, the negative controls (US off) showed (see Table 1) all a decrease in bacteria count; both systemically released gentamicin (Pa) and locally released gentamicin (PaG and PePaG) reduced the CFU. Out of these negative controls PaG showed the highest reduction in CFU.

Compared to the negative controls, application of ultrasound (US on) resulted in an enhanced killing of bacteria in seven out of nine experiments performed. The highest average percentage of reduction was seen for Pa with systemic gentamicin (69%), shortly followed by PePaG (64%) and PaG (58%).

Analysis after necropsy showed that within 48 h a well-organized, subcutaneous capsule of fibrin, heterophils and bacteria had formed around the implanted disks on both sides. Edema, congestion and inflammation were prominent, surrounding the capsule. Capsules around cement disks not subjected to ultrasound showed more signs of inflammation in all experimental groups: capsule formation was more extensive, capsules were larger and were mostly filled with larger volumes of pustular fluid. In general, these qualitative findings were in accordance with the reduced numbers of viable bacteria on cement disks treated with ultrasound in the experimental groups. No signs of specific skin degradation or necrosis were observed due to ultrasound in either group. Histopathological studies of the kidneys from one

rabbit in each experimental group showed no abnormalities, such as signs of nephrotoxicity, and none of the blood samples showed bacteremia.

DISCUSSION

Currently, no effective non-invasive technique exists to prevent or treat biofilm infections associated with medical implants. Efforts to develop a non-invasive, effective treatment for biofilm infections on medical implants have been undertaken and include electric and ultrasonic enhancement of antibiotics, coating of biomaterials with antibiotic, and embedding antibiotics in biomaterials, such as in acrylic bone cement (Buchholz and Engelbrecht 1970; Blenkinsopp *et al.* 1992; Schierholz *et al.* 1997; Johnson *et al.* 1998). Numerous studies demonstrated an effect of ultrasound in combination with an antibiotic on bacterial populations (Zips *et al.* 1990; Huang *et al.* 1996; Qian *et al.* 1997; Johnson *et al.* 1998; Qian *et al.* 1999; Peterson and Pitt 2000; Carmen *et al.* 2004a), but none of these studies involve ultrasound effects in combination with the controlled release of antibiotics from antibiotic-loaded bone cements.

This study showed that ultrasound in combination with gentamicin yielded a tendency towards enhanced bacterial killing in biofilms growing on acrylic bone cements in all groups. Ultrasound enhanced bacterial killing (in percentages, see last column of Table 1) was the largest for plain bone cement (Pa) in combination with the use of systemic antibiotics, although quantitatively (column of enhanced killing, see Table 1) the highest reduction was observed for post elution antibiotic-loaded-bone cement (PePaG) in the absence of systemic antibiotics. Although gentamicin release from PePaG is considered to be negligible (Van de Belt *et al.* 2000a; Torrado *et al.* 2001; Virto *et al.* 2003) and gentamicin release is not accelerated by ultrasound on post-elution samples in vivo (Hendriks *et al.* 2003), an enhanced bacterial killing by ultrasound could be observed. This shows that PePaG samples still release gentamicin resulting in enhanced killing of biofilm.

A limitation in the present experimental set-up might underestimate the (ultrasound) effects on bacterial killing for the group of PaG. Firstly, the elution characteristics of antibiotic-loaded bone cements show that the initial release burst of antibiotics from bone cement occurs in the first 6–10 h (Del Real *et al.* 2000, Van de Belt *et al.* 2000b). In the model used in this paper, a biofilm was seeded on bone cement in liquid media for 24 h; therefore the gentamicin release for the samples in the PaG group was lower than could be expected from newly implanted PaG. Nevertheless, this lower amount of gentamicin in the group of PaG that was released resulted in the highest reduction in the negative controls (US off), suggesting that local release of gentamicin can be considered to be more effective in reducing biofilm on bone cement than systemic administration (PaG) of gentamicin.

Secondly, because a bigger part of the original biofilm is already eradicated by the higher local gentamicin concentration, there is less biofilm to kill compared to the groups of Pa and PePaG. Remaining biofilm is known to be harder to eradicate. Nevertheless, the percentage enhanced killing by ultrasound remains on a level in which ultrasound reduces remaining biofilm with more than 50%.

Thirdly, after implantation, gentamicin that is released from PaG and PePaG spreads into a large volume of subcutaneous area, which is much larger than the small prosthesis-related gap (Hendriks *et al.* 2002) in which gentamicin is released from bone cements in clinical use. Consequently, a high percentages of gentamicin released from the PaG and PePaG samples are lost until fibrous capsule surrounds the samples. Larger bacterial reductions may therefore be expected in clinical usage, where loss of gentamicin is limited and smaller volumes apply, especially as aminoglycosides have a concentration dependent antibacterial activity (Lacy *et*

al. 1998). The previously finding of increased gentamicin release on PaG by ultrasound (Hendriks *et al.* 2003) may then be even more beneficial.

In conclusion, application of ultrasound resulted in an enhanced (>50%) bacterial killing in combination with systemic gentamicin and with gentamicin released from antibiotic-loaded bone cement in comparison with the negative control. These results suggest that application of ultrasound during the early postoperative period combined with usage of antibiotic-loaded bone cement or systemic antibiotics may contribute to the prevention of implant infection in future clinical practice.

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Average number of viable bacteria cultured from bone cement samples (Pa, plain bone cement in combination with systemic gentamicin; PaG, gentamicin-loaded cement in the absence of systemic antibiotics; PePaG, gentamicin-loaded cement after elution in the absence of systemic antibiotics) in the different experimental groups in the absence (US off) or presence (US on) of ultrasound in vivo. Enhanced killing by ultrasound was calculated as (CFU cm⁻², US off – CFU cm⁻², US on). Percentage enhanced killing represents the percentage reduction that is achieved by applying ultrasound; this was calculated as (1 - (US on / US off)) * 100.

Table 1

Bone cement	n	US off (CFU cm ⁻²)	US on (CFU cm ⁻²)	Enhanced killing (CFU cm ⁻²)	Percentage enhanced killing
Pa	3				
PaG	3	1.4 × 10 ⁷	4.3 × 10 ⁶	9.6 × 10 ⁶	69%
PePaG	3	3.6 × 10 ⁴	1.6 × 10 ⁴	2.0 × 10 ⁴	58%
		5.9 × 10 ⁷	2.1 × 10 ⁷	3.8 × 10 ⁷	64%