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Treatment of Biofilm Infections on Implants with Low-frequency Ultrasound and Antibiotics

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Summary

Medical implants are sometimes colonized by biofilm-forming bacteria that are very difficult to treat effectively. The combination of gentamicin and ultrasonic exposure for 24 h was previously shown to reduce the viability of *E. coli* biofilms in vivo. This article shows that such treatment for 48 h reduced viable *E. coli* bacteria to nearly undetectable levels. However, when *P. aeruginosa* biofilms were implanted and treated for 24 and 48 h, no significant ultrasonic-enhanced reduction of viable bacteria was observed. The difference in response of these two organisms is attributed to greater impermeability and stability of the outer membrane of *P. aeruginosa*.

Keywords

ultrasound; gentamicin; biofilm; implant infection; *E. coli*; *P. aeruginosa*; rabbit model

Introduction

Bacteria sequestered in biofilms exhibit increased tolerance to the normal antibiotic therapies. Although the mechanism of this resistance is not known, current hypotheses on the subject include the heterogeneity of biofilm-encased bacteria and the decreased penetration of antibiotics due to interactions with the exopolysaccharide matrix (1–4). Currently, the only effective treatment for biofilm infections is to remove the implant, fight the infection with antibiotics, and replace the implant, a costly and stressful procedure. To this end, our research group is pursuing alternative treatments. Previous in vivo experiments showed that gentamicin combined with 24 h of ultrasonic treatment resulted in the enhanced reduction of viable bacteria in *Escherichia coli* biofilms (5,6). In this article, we report the successful use of ultrasound in vivo when the treatment of *Escherichia coli* biofilms was extended to 48 h, and the failure of such therapy to reduce viable bacteria in *Pseudomonas aeruginosa* biofilms.

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Methods and Materials

Nineteen New Zealand White female rabbits were maintained under the regulations of the Institutional Animal Care and Use Committee of Brigham Young University and the U.S. Department of Agriculture. Five rabbits received *E. coli* biofilms and were treated for 48 h. Fourteen rabbits received *P. aeruginosa* biofilms including two that received neither ultrasound nor gentamicin, one that received ultrasound but no gentamicin, six that received gentamicin and ultrasound for 24 h, and five that received ultrasound and gentamicin for 48 h.

The biofilms were grown for 24 h on polyethylene disks as reported previously (5,6). *E. coli* ATCC 10798 and *P. aeruginosa* ATCC 27853 used in these experiments had planktonic MIC values of 6 and 3 ug/mL respectively. The MIC was determined upon visual inspection of cultures grown in increasing concentrations of antibiotic upon incubation at 37°C. The lowest concentration exhibiting no growth was designated as the MIC (7).

Rabbits were anesthetized and the infected disks implanted subcutaneously as reported previously. Briefly, two bilateral incisions were made perpendicular to the vertebral column. Implant sites were created using blunt scissors to tunnel just beneath the cutaneous trunci parallel to the vertebral column. Each infected disk was sutured to the underside of the skin through holes in sewing tabs on the disk, and the incision was sewn closed.

Gentamicin (8 mg/kg, Gentocin; Schering-Plough, Kenilworth, N.J.) was injected subcutaneously (SC) in the dorsal cervical region every 24 h commencing immediately after the incisions were closed. Concurrently the rabbits received Banamine (2mg/kg SC; flunixin-megalumine, Schering-Plough, Kenilworth, N.J.) in the contralateral cervical region to relieve pain or discomfort due to surgery. Blood was sampled daily from a marginal ear vein immediately before surgery and every 24 h thereafter just prior to the injection of gentamicin and Banamine. The blood samples were diluted and plated onto nutrient agar (NA) using membrane filtration.

The ultrasound was delivered using a 28.5 kHz ultrasound transducer (EDO Acoustics, Salt Lake City, UT). The transducer was placed over one of the implanted disks 24 h after surgery and held in place with a canvas jacket. The transducer was fixed onto the skin with an acoustically conductive gel adhesive (Tensive; Parker Laboratories) and was about 3 mm from the disk (through skin and fascia). Air was delivered to the transducer at about 62 cm^3/min to convectively transfer away heat. Pulsed ultrasound was delivered in a 1:3 duty cycle at an intensity of 500 mW/cm^2 during the pulse. Insonation commenced 24 h post surgery and was applied for either 24 or 48 h. Then the rabbits were euthanized using a 1-cc intravenous injection of a solution composed of 26% sodium pentobarbital and 7.8% isopropyl alcohol (Sleepaway, Ft. Dodge, Iowa).

The implanted disks were exposed and removed with sterile forceps, and the sewing tabs were cut from the disk using sterile scissors. The circular portion of the disk was placed in a test tube containing trypsin in PBS. Disks with *E. coli* biofilms were placed in 10 ml of 0.05% trypsin, whereas disks with *P. aeruginosa* biofilms were placed in 10 ml of 0.25% trypsin. The biofilms were stripped and dispersed using an ultrasonic cleaning bath as described previously. This treatment was previously shown not to decrease bacterial viability (8). Bacteria removed from the disk were quantified by serial dilution in PSS and membrane filtration using 0.45 um cellulose acetate filters that were placed on NA plates and incubated at 37°C for 48 h. The colonies were counted to determine the viable CFU/cm^2. The results were reported as $\log_{10}$ CFU/cm^2 plus or minus the standard deviation of the mean. The difference in the number of viable cells between biofilms treated with and without ultrasound was calculated and reported as “ultrasonic-enhanced reduction”.

At necropsy, the kidneys and heart were removed from one animal in each experimental group (*E. coli* or *P. aeruginosa*, 24-h or 48-h ultrasonic treatment) at the same time that the disks were removed. Histopathological examinations of these organs were performed by an independent lab (ARUP, Salt Lake City, UT) to detect any abnormalities.

Data were analyzed by one-tailed paired student t-test to evaluate the difference in $\log_{10}$ CFU/cm^2 recovered from implanted biofilms treated with gentamicin and from implanted biofilms treated with gentamicin and ultrasound. The data is presented in the form of box plots.

Results and Discussion

E. coli

Prior to implantation the E. coli biofilm viability was 6.80 ± 0.47 $\log_{10}$ CFU/cm^2. After 72 h of treatment with gentamicin alone, the number of viable bacteria was reduced to 2.29 ± 0.40 $\log_{10}$ CFU/cm^2, while treatment with both gentamicin (for 72 h) and ultrasound (for 48 h) reduced the viable bacteria to 0.011 ± 1.02 $\log_{10}$ CFU/cm^2 (Fig. 1). Combining ultrasound and gentamicin resulted in a statistically significant ultrasonic-enhanced reduction of 2.28 ± 0.83 $\log_{10}$ CFU/cm^2 ($p = 0.002$, n=5).

Histopathological studies of the organs and the insonated skin removed after euthanasia revealed no abnormalities. None of the blood samples showed any evidence of bacteremia.

P. aeruginosa

Prior to implantation, the *P. aeruginosa* biofilm viability was 7.60 ± 0.26 $\log_{10}$ CFU/cm^2. The two rabbits that received neither ultrasound nor gentamicin died within 24 h post surgery. Another rabbit without antibiotic commenced ultrasound treatment, but died after insonation commenced, about 36 h post surgery. Because of the high mortality without antibiotic, these control experiments were aborted in the interest of humane treatment of animals. The deaths also illustrate the severity of these infections. In comparison, all rabbits without gentamicin implanted with *E. coli* biofilms survived for the duration of the experiments (5).

Treatment with gentamicin reduced the number of viable *P. aeruginosa* to 3.86 ± 0.57 $\log_{10}$ CFU/cm^2 48 h post surgery and 4.04 ± 0.16 $\log_{10}$ CFU/cm^2 72 h post surgery (corresponding respectively to 24 and 48 h of ultrasound exposure on the contralateral side).

Treatment with both gentamicin and ultrasound for 24 h reduced the number of viable bacteria to 3.64 ± 0.68 $\log_{10}$ CFU/cm^2 (Fig. 2). This did not result in a significant ultrasonic-enhanced reduction in biofilm viability (enhanced reduction = 0.22 ± 0.51 $\log_{10}$ CFU/cm^2; $p = 0.17$).

When treated with gentamicin and 48 h of ultrasound, biofilm viability was reduced to 4.15 ± 1.18 $\log_{10}$ CFU/cm^2. As in the 24-h experiments, this combined treatment failed to significantly enhance the killing of *P. aeruginosa* in the biofilm (ultrasonic-enhanced reduction = -0.11 ± 1.12 $\log_{10}$ CFU/cm^2; $p = 0.588$).

Neither 24-h treatment nor 48-h ultrasonic treatment resulted in bacteremia. Moreover, histopathological examination did not detect abnormalities in the kidneys, the heart, and the insonated skin removed from the rabbit.

Currently, no effective, non-invasive technique exists to treat the biofilm infections associated with medical implants. Efforts to develop a non-invasive and effective treatment for biofilm infections on medical implants include electric and ultrasonic enhancement of antibiotics, and antibiotics applied onto or into biomaterials (6,8–14).

This model of an implant infection in a rabbit simulates a situation involving a well-developed infection on an implant. Ultrasound is applied 24 h post surgery to allow the rabbit to mount a response to the infection. At 24 h a fibrous capsule has formed around the implant and there is general inflammation in the region of the capsule. The ultrasound and antibiotic are applied at a time when the host has sequestered but not eliminated the infection. In this manner the model simulates a chronic implant infection.

The ultrasonic enhancement of antibiotic action has shown promise in vivo against organisms often found associated with implant infections. For example with biofilms of gram-positive *Staphylococcus epidermidis*, the application of ultrasound during vancomycin therapy further decreased the viability of the biofilm in vivo (15).

The therapy also works on gram-negative biofilms of *E. coli*. Previously we reported enhanced gentamicin activity to treat *E. coli* biofilms when continuous or pulsed ultrasound (in a 1:3 duty cycle with a pulse intensity of 500 mW/cm^2) was applied for 24 h. The rabbit's own immune system (without application of antibiotic) reduced the biofilm viability by 2 orders of magnitude, and when ultrasound was applied without application of antibiotics, there was no further change in biofilm viability (5). Gentamicin therapy in the absence of ultrasound reduced the biofilm viability by an additional order of magnitude. Most importantly ultrasonic treatment combined with gentamicin for 24 h reduced the number of viable bacteria to 0.79 $\log_{10}$ CFU/cm^2 compared to 2.74 $\log_{10}$ CFU/cm^2 when insonation was not applied (6).

Building upon these previous experiments, the length of treatment was extended to 48 h to determine whether the implant infection could be eliminated in vivo. Treatment with gentamicin and 48 h of ultrasound reduced the number of viable bacteria in the biofilms to an average of 1 CFU/cm^2. Thus increasing insonation from 24 h to 48 h reduced the number of viable bacteria from 0.79 $\log_{10}$ CFU/cm^2 bacteria to 0.011 $\log_{10}$ CFU/cm^2. These values are only marginally different from each other ($0.05 < p < 0.1$). The lack of difference at the 0.05 level can be attributed to two characteristics of the data collected. First, only six disks were implanted for the 24 h experiments; three that were treated with gentamicin alone and three that were treated with gentamicin and ultrasound. Increasing the sample size would probably result in a smaller p-value and a more significant difference. The second and more likely cause of the large p-value is the difficulty in detecting viable bacteria in the biofilms after 48 h of treatment with ultrasound. As Fig. 1 illustrates, the variation of the data was large. For example, no bacteria were recovered from one implant and 20 bacteria were recovered another implant. As the number of bacteria approaches zero, small variations lead to larger standard deviations. In all likelihood, an increase in sample size would illustrate more significantly the effect of extending the treatment of implanted *E. coli* biofilms to 48 h.

In our attempt to extend this therapy to a third and more notorious infectious organism, ultrasound failed to enhance killing of *P. aeruginosa* biofilms, even though the MIC of planktonic *P. aeruginosa* is less than that of *E. coli*. In contrast, in vitro application of ultrasound consistently enhanced the biocidal activity of aminoglycosides against planktonic and biofilm cultures of *P. aeruginosa* (3,7,16,17). The inability of ultrasound to enhance aminoglycoside activity in vivo against *P. aeruginosa* biofilms is surprising given the success against in vivo biofilms of *E. coli*. This difference may be related to the documented difference in outer membrane permeability between these bacteria (18–20). Previous in vitro studies showed that a 24-h planktonic cultures of *E. coli* were more susceptible to treatment with 12 μg/ml gentamicin and ultrasound than were 24-h planktonic cultures of *P. aeruginosa* (7).

Another difference in these two bacteria is that *E. coli* is facultatively anaerobic (21) while *P. aeruginosa* is classified as an obligate aerobe; however, *P. aeruginosa* can respire anaerobically using nitrate, nitrite or N_2O as the terminal electron acceptor (22,23). Oxygen

limitation may not be an issue during in vitro experiments, but it may become significant in the poorly vascularized subdermal pocket in the rabbit model. Aminoglycosides are more effective against metabolically active bacteria (24), and *E. coli* may be more active in this in vivo model. We postulate that the decreased membrane permeability of the *P. aeruginosa* or the less aerobic environment in vivo could be responsible for the lack of ultrasound enhanced gentamicin activity.

As to the clinical relevance of this work, the relatively recent recognition of the role of biofilms in multiple pathologies and the difficulty in resolving these pathologies speaks to the importance of developing means of replacing or enhancing the therapies already in use. The use of synthetic materials in the body ranges from catheters to mesh to stents to heart valves and beyond. Until the development of viable and practical tissue engineering, then number and types of applications in which synthetic materials are used will continue to increase. While it is of no consequence to remove and replace a urinary catheter, the replacement of an infected heart valve presents a much more daunting obstacle.

The use of ultrasound to enhance the activity of antibiotics is promising for several reasons. First, it makes use of two extant and ubiquitous clinical methodologies. Ultrasound used in multiple applications, is well accepted, and has few if any side effects. Antibiotics have been in use for over fifty years and have been well characterized. Second, ultrasound therapy allows for the treatment of biofilm infected surfaces in an non-invasive manner. Third, using ultrasound, the biofilm-infected device can be specifically targeted.

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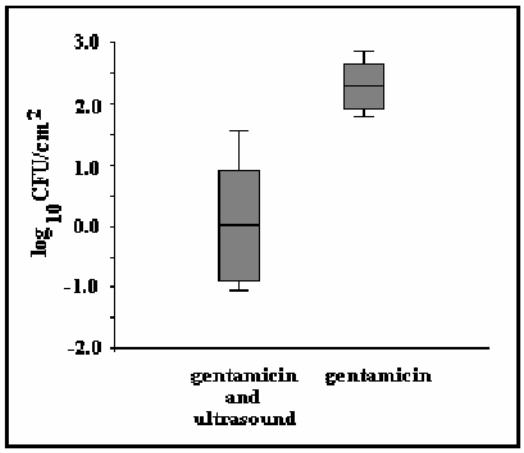

Figure 1.
Viable counts of *Escherchia coli* recovered from infected disks explanted from the rabbit model. The box plot on the left represents the data from disks exposed to 72 h of gentamicin, the last 48 h of which were accompanied by exposure to pulsed ultrasound. The box plot on the right represents the data from disks exposed to 72 h of gentamicin without any ultrasonic exposure. The top and bottom of the boxes represent the 75th and 25th percentiles and the median is indicated as the line in the box. The upper and lower adjacent values are represented by cross bars.

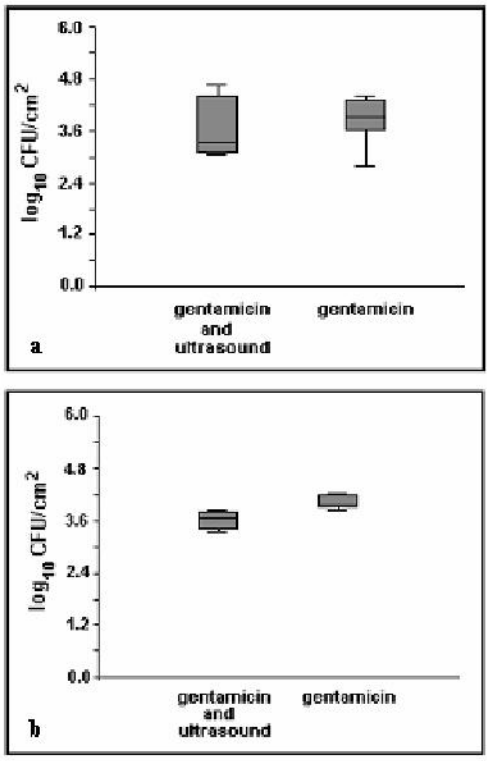

Figure 2.
Viable counts of *Pseudomonas aeruginosa* recovered from infected disks explanted from the rabbit model. The upper plot (a) represents data from rabbits exposed to ultrasound for 24 h, while the lower plot (b) represents data from rabbits exposed to ultrasound for 48 h. See the caption of Fig. 1 for details to the box plots.