Effect of Delayed Pulsed-Wave Ultrasound on Local Pharmacokinetics and Pharmacodynamics of Vancomycin-Loaded Acrylic Bone Cement In Vivo

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This study sought to investigate the effect of delayed pulsed-wave ultrasound with low frequency on drug release from and the antimicrobial efficacy of vancomycin-loaded acrylic bone cement in vivo and the possible mechanism of this effect. After the implantation of cement and the inoculation of Staphylococcus aureus into the bilateral hips of rabbits, ultrasound (average intensity, 300 mW/cm²; frequency, 46.5 kHz; on/off ratio, 20 min/10 min) was applied to animals in the normal ultrasound group (UG0–12) from 0 through 12 h after surgery and to those in the delayed-ultrasound group (UG12–24) from 12 through 24 h after surgery. The control group (CG) was not exposed to ultrasound. Based on vancomycin concentrations in left hip cavities at projected time intervals, the amount of time during which the local drug concentration exceeded the MIC (T>MIC) in UG12–24 was significantly prolonged compared with that in either CG or UG0–12, and the ratios between the areas under the concentration-time curves over 24 h and the MIC for UG0–12 and UG12–24 were both increased compared with that for CG. The greatest reductions in bacterial densities in both right hip aspirates and right femoral tissues at 48 h were achieved with UG12–24. Local hemorrhage in rabbits of UG0–12 during the 12-h insonation was more severe than that in rabbits of UG12–24. Of four variables, the T>MIC and the bioacoustic effect were both identified as parameters predictive of the enhancement of the antimicrobial efficacy of cement by ultrasound. Sustained concentrations above the MIC replaced early high maximum concentrations and long-term subtherapeutic release of the drug, provided that ultrasound was not applied until local hemorrhage was relieved. The enhancement of the antimicrobial efficacy of cement by ultrasound may be attributed to the prolonged T>MIC and the bioacoustic effect caused by ultrasound.

Of the patients worldwide undergoing total joint replacement per year, approximately 0.3 to 2.2% develop prosthesis-related infections resulting in devastating surgical failure (23, 25). Antibiotic-loaded bone cement is the treatment of choice because of its high local dose and low systemic toxicity compared with those of intravenous antibiotics (23, 25). However, it is sometimes deficient in antimicrobial efficacy (21). Many authors have attributed this defect to the incomplete release of the antibiotic from the cement (4, 5, 6, 8, 20, 23, 24). The matrix of polymethylmethacrylate is, to a large extent, impermeable to antibiotics. Not only is the bioavailability of the antibiotic decreased, but the prolonged exposure to the antibiotic also allows selective bacterial resistance to occur (20).

Recently, low-frequency ultrasound has been found to enhance the release of gentamicin from cement (4, 8). Two possible mechanisms behind this phenomenon include acoustic streaming and an accelerated rate of mass transfer as a result of stable cavitation and the ultrasonic pressure wave. Also, such enhanced release of gentamicin may contribute to a decrease in the viability of bacteria (5, 6). In our previous in vitro study (24), we determined that intermittent continuous-wave ultrasound with a low intensity could increase drug elution from vancomycin-loaded acrylic bone cement during the stage of low-level drug release. In our parallel animal study (24), however, the drug level in hip cavities sank sharply to concentrations below the MIC after the concentration peak initiated by the first insonation. As a result, the length of time during which the local drug concentration exceeded the MIC (T>MIC) was not prolonged with intermittent insonations, which possibly did not contribute to the enhanced antimicrobial efficacy of cement in vivo, as vancomycin exhibits time-dependent killing of bacteria (19). We postulate that either the transmission mode of ultrasonic energy or the severe hemorrhage and hyperemia of the hip in the early postoperative period may be responsible for the lack of a prolonged T>MIC in vivo.

In the present study, we developed three sequential hypotheses: (i) pulsed-wave ultrasound may increase the drug release from cement more than continuous-wave ultrasound; (ii) delayed ultrasound may prevent the severe dilution and absorption of local vancomycin in the early postoperative period and, thus, effectively prolong the T>MIC; and (iii) a prolonged T>MIC and antimicrobial synergism between vancomycin and ultrasound (1) are two possible causes of the enhancement of the antimicrobial efficacy of cement by ultrasound. Consequently, the present investigation focused on the pharmacokinetics (PK) and pharmacodynamics (PD) of antibiotic-loaded...
When applied with ultrasound, the influence of delayed application on the ultrasonic effect, and their possible mechanisms.

**MATERIALS AND METHODS**

**Bone cement.** The diameters and heights of the cement specimens were determined based on the measurement of femur dimensions in rabbits by using radiographs. Cement specimens were prepared by adding powdered vancomycin (Vancocin CP; Eli Lilly, United States) to 40 g of bone cement (CMW Endurance; DePuy, England). With the procedure described previously (24), cylinder specimens (3 mm in diameter, 30 mm in length, and weighing 0.318 ± 0.017 g) were manufactured and stored at 4°C (Fig. 1).

**Microorganisms.** *Staphylococcus aureus* ATCC 13565 (Biological Authentication Research Institute, China) was reconstituted from stock frozen at −80°C, incubated in Mueller-Hinton broth (Oxoid, Basingstoke, England) overnight, and diluted approximately 2 h before inoculation to produce a suspension of 10^8 CFU/ml. The MIC and the minimum bactericidal concentration of vancomycin for this planktonic strain were determined to be 2 and 3 μg/ml, respectively, by a tube dilution technique with rabbit serum as the medium (3).

**Implantation and inoculation.** Twenty-eight healthy adult female New Zealand and White rabbits (Animal Center of Zhejiang University, China), with an average weight of 2.50 ± 0.28 kg, were obtained 7 days before surgery to be acclimatized to the Clinical Animal Laboratory at the Second Affiliated Hospital of Zhejiang University, Zhejiang, China. The experimental protocol was approved by the animal ethics committee of Zhejiang University. International laws and regulations for medical research with experimental animals were followed.

Experimental acute infection in hips and femurs was established with the same procedures for both sides under sterile conditions. After anesthesia was administered (24), the hip cavity was exposed. Subsequently, the femoral head was dislocated and cut out. A small suction tube was inserted into the femoral canal to remove as much blood and bone marrow as possible. Thereafter, a cement specimen was inserted with its end 8 mm above the cutting plane. Then the hip joint was reduced. A 4-mm-diameter hole was drilled into the proximal femur 3 cm from the proximal end of the femur (Fig. 2). One hundred microliters of sodium morrhuate, followed by 100 μl of a suspension containing 10^7 CFU of *S. aureus*, was injected into the proximal femoral canal. The hole was sealed with surgical wax. A silicon drain was placed at the proximal end of the cement specimen. The muscles were rejoined, as was the skin. Finally, 1 ml of a suspension containing 10^6 CFU of *S. aureus* was injected into the hip cavity. The animals lost 5.84 ± 4.35 ml of blood without receiving an intravenous infusion or prophylactic antibiotic postoperatively. The postoperative rehabilitation and pain relief procedures were the same as those described in our previous study (24).

**Grouping and insonation.** All 28 rabbits were randomly divided into three groups: (i) the control group (CG; n = 8) with transducers fixed over bilateral

![Flow chart of the study](http://aac.asm.org/Downloaded from October 14, 2017 by guest)
Ohmic, United States) to calibrate the power intensity of 900 mW/cm², which was pulsed by a 3.0-cm-diameter unfocused transducer. The frequency was produced a sinusoidal wave at 46.5 kHz. Serial 10-fold dilutions were prepared, and 100 μl of each dilution was plated onto tryptic soy broth (Oxoid, Basingstoke, England). After a 48-h incubation in 5% CO₂ at 37°C, viable bacteria in each dilution were counted and the numbers were multiplied by the dilution coefficient. The S. aureus bacterial concentration (the bacterial density) in each sample was calculated as the log₁₀ number of CFU per milliliter of hip aspirate or per gram of femoral tissue. Because the defined enhanced reduction as the difference in bacterial density between CG samples and samples from either UG₀–₁₂ or UG₁₂–₂₄. This reduction significantly enhanced the antimicrobial efficacy of cement produced by ultrasound (1). Isolates from all positive cultures were identified by colony morphologies, Gram staining, and catalase and coagulase tests (24).

Quantitative bacterial cultures of both hip aspirate and femoral tissue were performed in triplicate. Serial 10-fold dilutions were prepared, and 100 μl of each dilution was plated onto tryptic soy broth (Oxoid, Basingstoke, England). After a 48-h incubation in 5% CO₂ at 37°C, viable bacteria in each dilution were counted and the numbers were multiplied by the dilution coefficient. The S. aureus bacterial concentration (the bacterial density) in each sample was calculated as the log₁₀ number of CFU per milliliter of hip aspirate or per gram of femoral tissue. We defined the enhanced reduction as the difference in bacterial density between CG samples and samples from either UG₀–₁₂ or UG₁₂–₂₄. This reduction significantly enhanced the antimicrobial efficacy of cement produced by ultrasound (1). Isolates from all positive cultures were identified by colony morphologies, Gram staining, and catalase and coagulase tests (24).

Statistical analysis. Based upon Lehr’s formula (12), the optimal sample size for each group in the PK and PD studies was determined to be eight. Data were expressed as means ± standard deviations. After a homogeneity test was performed and a test for Gaussian distribution were both passed, Student’s unpaired t test was performed to determine the significance of variation in the volumes of sera from hip cavities of rabbits of UG₀–₁₂ and UG₁₂–₂₄. All other data for the three groups were compared using one-way analysis of variance with the least significant difference (LSD) test for multiple comparisons.

The PK and PD results for the three groups were pooled together to set up a multiple linear regression model. This model allowed a comprehensive evaluation of possible causes of different levels of antimicrobial efficacy among groups. The dependent variable was the bacterial density in the hip aspirate after 48 h. Independent variables included the Cₘₐₓ/MIC ratio, the Tₘₐₓ (in hours), the AUC₂₄/MIC ratio, and the status of antimicrobial synergism between ultrasound and released vancomycin (4) (coded as two dummy variables: for CG, dummy 1 was 0 and dummy 2 was 0; for UG₀–₁₂, dummy 1 was 1 and dummy 2 was 0; for UG₁₂–₂₄, dummy 1 was 1 and dummy 2 was 1; i.e., the dummy 1 in both ultrasound groups was 1). After the stepwise removal of insignificant variables, the best linear regression model was constructed. Finally, the assumption of normality and the spread of the residue for the best model were evaluated. A statistically significant difference was set as a P value of <0.05.

RESULTS

PK study. Figure 3 shows the profiles of local drug release for the three groups during 48 h after surgery. All release curves showed bimodal profiles consisting of a transient initial peak followed by a long-term slow release. The Cₘₐₓ was reached at 3 h for CG, 2 h for UG₀–₁₂, and 3 h for UG₁₂–₂₄, with the highest Cₘₐₓ being that for UG₀–₁₂. Thereafter, the local drug levels in each group dropped sharply. For CG and UG₀–₁₂, the mean drug concentration dropped below the MIC at 15 h. Noticeably, for UG₁₂–₂₄, there was a slight rise at 12 h, when ultrasound started, followed by the maintenance of a steady drug level above the MIC for 24 h.

Locally hemorrhage. The total volume of drainage fluid from UG₀–₁₂ during the 12-h period of insonation was 4.65 ± 1.42 ml, which was significantly higher than the volume from UG₁₂–₂₄ of 1.32 ± 0.35 ml (t = 4.566; P < 0.01).

PD study. During quantitative cultures of hip aspirates and femoral tissues from the three groups, we identified all the grown colonies as the inoculated S. aureus strain without observing any other bacteria. The bacterial densities in hip aspirates and femoral tissues at 48 h after surgery are shown in Fig.

FIG. 2. A lateral X-ray of the right (R) femur of a rabbit shows that a cement specimen (indicated by the asterisk) was inserted into the proximal femur and a 4-mm-diameter hole was drilled on the backside of the proximal femur (indicated by the white arrow). Also, a drainage tube was placed into the periprosthetic space. Hip aspirates were drained through the drainage tube in the hip cavity at projected time intervals.
The enhanced reduction in hip aspirates from UG 0–12 was log$_{10}$ 1.62 CFU/ml ($P$/H11021 0.05), and that in hip aspirates from UG12–24 was log$_{10}$ 2.77 CFU/ml ($P$/H11021 0.01). The enhanced reduction in femoral tissues from UG 12–24 was log$_{10}$ 1.29 CFU/g ($P$/H11021 0.05); however, the enhanced reduction in tissues from UG0–12 was not statistically significant (log$_{10}$ 0.53 CFU/g; $P$ = 0.39).

PK/PD analysis. Three PK/PD parameters for the three groups are shown in Table 1. The multiple linear regression model (Table 2) demonstrated that neither the $C_{\text{max}}$/MIC ratio nor the $AUC_{24}$/MIC ratio was significant in predicting the antimicrobial efficacy of the combination of cement and ultrasound. After the stepwise removal of two insignificant variables, the best linear regression model (Table 2) showed a
satisfactory prediction of the results with an adjusted $R^2$ (coefficient of determination) of 0.907 ($F = 65.889$; $P < 0.001$).

Three variables, the $T_{\text{MIC}}$, dummy 1, and dummy 2, were all significant predictor variables. The regression coefficients for the $T_{\text{MIC}}$ and dummy 1 were both negative, indicating that the prolongation of the $T_{\text{MIC}}$ and antimicrobial synergism between ultrasound and released vancomycin both played a positive role in reducing the bacterial densities in hip aspirates. The verification of the normality and the spread of the residue for the best model was satisfactory.

**DISCUSSION**

From the perspective of physical therapy, pulsed-wave ultrasound is suggested to be preferable to continuous-wave ultrasound by virtue of a higher power intensity and a lower chance of tissue burning. In our previous study in vivo (24), continuous-wave ultrasound failed to prolong the $T_{\text{MIC}}$ in spite of the increased vancomycin release. In the present study, the results for UG0–12 indicated that vancomycin release enhanced by pulsed-wave ultrasound followed a profile similar to that of vancomycin release in our previous study (24), which accounted for the lack of a prolonged $T_{\text{MIC}}$ for UG0–12. Our first hypothesis, that pulsed-wave ultrasound, was superior to continuous-wave ultrasound, was not confirmed. Certainly, a low intensity, a short exposure time, and a small amount of loaded vancomycin may be of influence. Further comparative studies will be needed to address the role of the mode of transmission of ultrasonic energy in enhanced drug release from cement.

The release kinetics for CG showed that the local drug level sank below the MIC at 12 h after surgery. Accordingly, the insonation of UG12–24 was delayed until 12 h after surgery in order to produce a continuous drug level above the MIC and a resulting decrease in viable bacteria. The PK for UG12–24 showed a prolonged $T_{\text{MIC}}$ and a drug level marginally above the MIC for 36 h, which was strikingly different from the results for UG0–12. Two mechanisms may possibly contribute to the difference. (i) The level of local hemorrhage caused by surgical trauma in UG0–12 from 0 through 12 h was approximately 3.5 times that in UG12–24 from 12 through 24 h. Such severe hemorrhage inevitably diluted local drug concentrations. (ii) As local hemorrhage was more severe in UG0–12 than in UG12–24, the area of contact between vancomycin and hip tissue in UG0–12 was possibly larger. Furthermore, the local blood circulation in UG0–12 became more active as a result of a trauma-induced stress reaction in the early postoperative period. Therefore, UG0–12 might have had greater absorption, distribution, and elimination of vancomycin than UG12–24. Our PK data confirmed our second hypothesis. In view of a $T_{\text{MIC}}$ for UG12–24 more than double that for CG and a highly reduced $T_{\text{MIC}}$ for CG compared with human data (11, 14), we postulate that the delayed-ultrasound-enhanced $T_{\text{MIC}}$ for humans will probably far exceed 36 h.

All the conclusions in the literature concerning the value of PK/PD parameters for predicting antimicrobial efficacy are based on systemic antibiotics (19) and, thus, are not necessarily applicable to antibiotic-loaded cement, since the local drug concentration differs greatly from the serum drug concentration (22). Our data from regression analysis demonstrated that of three PK/PD parameters, only the $T_{\text{MIC}}$ significantly correlated with the residual bacterial density in hip aspirate, which was consistent with the time-dependent characteristics of vancomycin. Our results may facilitate a better understanding and prediction of the local antimicrobial efficacies of various systems of antibiotic delivery which have been suggested for improving or replacing the erratic drug release from cement (10, 11, 13, 15, 23, 24).

Pitt and coworkers (1, 2, 16, 17, 18) found antibacterial synergism between antibiotics and ultrasound, or the bioacoustic effect. This effect was attributed to a sonoporation-induced inflow of the antibiotic through a membrane. The present study showed an enhanced reduction in the bacterial densities in samples from UG12–24, a trend towards enhanced reduction in samples from UG0–12, and a significant prediction of the bioacoustic effect on enhanced reduction, which were consistent with results previously reported in the literature. As extremely high concentrations of antibiotic may be reached in the prostheses-related interfacial gap (9), vancomycin concentrations above the MIC in the femoral canals of the three groups might have been maintained throughout the 48-h duration. Moreover, as the ultrasound intensity in the femoral canal may be less than 100 mW/cm² (7), ultrasound possibly neither inhibited bacteria (16) nor promoted drug release from cement (24). Therefore, we attributed the enhanced reduction in the femoral canals of UG12–24 to the bioacoustic effect instead of enhanced drug release or the direct killing of bacteria by ultrasound, which supported our third hypothesis. The bioacous-

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### TABLE 1. Three PK/PD parameters of local vancomycin release from hip cavities of rabbits of CG, UG0–12, and UG12–24 and one-way analysis of variance of PK/PD parameters among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>$C_{\text{MAX}}$/MIC</th>
<th>$T_{\text{MIC}}$ (h)</th>
<th>AUC$_{24}$/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>5.94 ± 0.97</td>
<td>14.89 ± 4.22</td>
<td>22.12 ± 6.61</td>
</tr>
<tr>
<td>UG0–12</td>
<td>14.72 ± 2.30</td>
<td>13.59 ± 1.57</td>
<td>38.40 ± 7.69</td>
</tr>
<tr>
<td>UG12–24</td>
<td>5.63 ± 1.81</td>
<td>35.40 ± 5.94</td>
<td>40.71 ± 13.31</td>
</tr>
</tbody>
</table>

**F value**: 60.06, **P value**: <0.001

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### TABLE 2. Multiple linear regression model and best multiple linear regression model values for the effects of predictor variables on bacterial densities in hip aspirates

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>Multiple linear regression model/best multiple linear regression model</th>
<th>Regression coefficient (SE)</th>
<th>t value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td></td>
<td>8.928/8.286 (0.549/0.369)</td>
<td>16.252/22.474</td>
<td>&lt;0.001/0.001</td>
</tr>
<tr>
<td>$C_{\text{MAX}}$/MIC</td>
<td></td>
<td>-1.021/NA (0.088/NA)</td>
<td>-1.152/NA</td>
<td>0.267/NA</td>
</tr>
<tr>
<td>$T_{\text{MIC}}$</td>
<td></td>
<td>-0.215/0.134 (0.044/0.023)</td>
<td>-4.662/5.942</td>
<td>&lt;0.001/0.001</td>
</tr>
<tr>
<td>AUC$_{24}$/MIC</td>
<td></td>
<td>0.026/NA (0.013/NA)</td>
<td>0.208/NA</td>
<td>0.061/NA</td>
</tr>
<tr>
<td>Dummy 1*</td>
<td></td>
<td>-1.867/1.794 (0.394/0.230)</td>
<td>-3.145/7.793</td>
<td>0.007/0.001</td>
</tr>
<tr>
<td>Dummy 2*</td>
<td></td>
<td>0.632/1.787 (0.593/0.547)</td>
<td>2.660/5.267</td>
<td>0.018/0.005</td>
</tr>
</tbody>
</table>

* Data are means ± standard deviations.
* $P < 0.01$ in LSD test for multiple comparisons with two other groups.
* $P < 0.01$ in LSD test for multiple comparisons with CG.

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* The variables $C_{\text{MAX}}$/MIC and AUC$_{24}$/MIC were both excluded from the best model. NA, not applicable.

* Dummy variables 1 and 2 were used to code for the status of antimicrobial synergism between ultrasound and vancomycin. Dummies 1 and 2 were 0 and 0 for CG, 1 and 0 for UG0–12, and 1 and 1 for UG12–24, respectively.
tic effect may provide promise for the noninvasive treatment of intraosseous infections, including prosthesis-related infections.

As control groups with drug-free bone cement or with ultrasound alone in our preliminary study in vivo presented high mortality (2), such groups were not included in the present study. We acknowledged certain limitations in the present study. (i) The ultrasonic durations for both UG0–12 and UG12–24 were set at 12 h. Further studies are required to reveal the saturation point, or the length of time after which the ultrasonic effect is no longer significant. (ii) The difference in the absorption, distribution, and elimination of vancomycin between UG0–12 and UG12–24 could not be clarified because of undetectable vancomycin levels and the noncompliance of animals in urination. (iii) The ratio between the cement size and the periprosthetic space was far smaller than that for a human (11). The present protocol may underestimate the enhancement of the PK and PD of cement by delayed ultrasound. (iv) The antimicrobial efficacy of the combination of cement and ultrasound on biofilms and its cellular and molecular mechanisms deserve further investigation.

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