In Vivo Assessment of the Antimicrobial Activity of a Calcium-Deficient Apatite Vancomycin Drug Delivery System in a MRSA Rabbit Osteomyelitis Experimental Model

G. AMADOR\textsuperscript{1,2}, H. GAUTIER\textsuperscript{3}, V. LE MABECQUE\textsuperscript{2}, A.F. MIEGEVILLE\textsuperscript{2}, G. POTEL\textsuperscript{1,2}, J.M. BOULER\textsuperscript{3}, P. WEISS\textsuperscript{3}, J. CAILLON\textsuperscript{1,2*}, C. JACQUELINE\textsuperscript{2}.

\textsuperscript{1}CHU de Nantes, \textsuperscript{2}Université de Nantes, faculté de Médecine,UPRES EA3826, UFR de Médecine 1 rue Gaston Veil Nantes, 44035 France, \textsuperscript{3}INSERM UMRS 791, Laboratory of Osteoarticular and Dental Tissue Engineering 1, place Alexis Ricordeau, Nantes 44042, France.

*Corresponding author. Mailing address : EA 3826, Faculte de Médecine, 1 rue Gaston Veil, 44035 Nantes, France. Phone and fax : (33) 240-412-854. E-mail : jocelyne.caillon@univ-nantes.fr.
Abstract

Antimicrobial activity of calcium-deficient apatite loaded with different concentrations (25, 100 and 500 µg/mg) of vancomycin was evaluated as a filling biomaterial in a MRSA rabbit acute osteomyelitis model. Bacterial counts in bone, bone marrow, and joint fluid treated with forms of the apatite were compared to tissue receiving a constant intra-venous vancomycin infusion after 4 days. This study demonstrates that calcium-deficient apatite loaded with vancomycin dramatically decreases the bacterial counts in bone and marrow.
Calcium-deficient apatites (CDA) are mineral groups lacking calcium, but similar in structure and function to those biological components found in bone and other calcified tissues. These synthetic matrices have osteoconductive properties and generate processes of resorption-substitution, which serves to quicken the healing process (2). CDA, as a filling biomaterial, can be loaded with an antimicrobial drug, vancomycin, to form a drug-delivery system used in osteomyelitis treatment (8). Osteomyelitis is characterized by severe bone loss, secondary to an acute or chronic inflammatory response caused by a bacterial invasion (1,5). Bone infections sometimes require surgical debridement, followed by extensive antibiotic treatment (5). *Staphylococcus aureus* is the most common form of bacteria found in osteomyelitis patients and as such, has become increasingly resistant to the often prescribed antibiotic treatment, antistaphylococcal penicillins (4,9,10). The options for treatment of bone infections due to methicillin-resistant *S. aureus* (MRSA) are limited by pharmacokinetic factors (such as penetration into bone tissues). At least 6 weeks of parenteral therapy are usually needed to reach efficient concentrations in situ (3). Among the glycopeptides, vancomycin is considered only as a reference molecule but is restricted to intravenous administration (4,11). Bolus injection is not possible, resulting in the use of prolonged infusion over at least an hour or, more recently, continuous infusion. If continuous infusion is used, serum steady-state concentration of approximately 20 to 25 times the minimum inhibitory concentration (MIC) should be targeted. Studies show that these values are often required in MRSA severe infections (11). The aim of this work was to assess the *in vivo* activity of CDA loaded with vancomycin versus constant infusion of vancomycin in an acute osteomyelitis model.

Female New Zealand white rabbits (weight: 2.0 to 2.5 kg) were anesthetized with intramuscular ketamine and xylazine, and experimental osteomyelitis was established in the distal right femur. A Jamshidi bone marrow biopsy needle was inserted between the two femoral
condoyles through epiphysis, physis and metaphysis, to reach the medullary canal. The Staphylococcus aureus strain used in this study was a MRSA strain isolated from a blood culture with a MIC of 1 µg/mL to vancomycin. One milliliter of a $10^9$ CFU/mL bacterial suspension was injected into the knee cavity. Animals were randomly assigned to nine different treatment groups: vancomycin group (VIV) (vancomycin constant IV infusion to reach a 20x MIC serum steady-state concentration), CDA alone (CDA), CDA+V (CDA unloaded in addition to vancomycin constant infusion), CDA25 (CDA loaded with 25 µg/mg of vancomycin), CDA25+V (CDA loaded with 25 µg/mg in addition to vancomycin constant infusion), CDA100 (CDA loaded with 100 µg/mg of vancomycin), CDA100+V (CDA loaded with 100 µg/mg of vancomycin in addition to vancomycin constant infusion), CDA500 (CDA loaded with 500 µg/mg of vancomycin in addition to vancomycin constant infusion). CDA loaded were made to release 80% of the total vancomycin introduced by wet granulation over the first 4 days. The release was monitored and verified through HPLC assays. On day 3, joint fluid (JF), femoral bone marrow (BM) and epiphyseal bone samples (BO) were obtained, placed immediately on ice, weighed, homogenized in 0.5 mL of saline buffer, and plated on trypticase-soja agar and Chapman plates using a Spiral® System (Interscience, St Nom La Bretèche, France). Afterward, a surgical debridement of the infected tissues and a rinse with sterile saline solution were performed. Aliquots of 100 mg of CDA were introduced in the femoral osseous gap for CDA, CDA+V, CDA25+V, CDA25, CDA100, CDA100+V, CDA500 and CDA500+V groups. Constant infusion of vancomycin started at day 3 and continued for 4 days for the VIV, CDA+V and CDA25+V, CDA100+V, and CDA500+V groups. On day 7, animals were euthanized by a lethal intravenous bolus of thiopental, and joint fluid, bone marrow, and bone were collected. Dilutions at $10^{-2}$ and $10^{-4}$ of the samples were made to eliminate potential carry-over effects. Surviving bacteria in JF, BM and BO were counted.
after a 48-h aerobic incubation at 37°C. The counts were normalized in the form log_{10}/g and compared to day 3 data. Results were expressed as the difference between log_{10}/g day 3 and log_{10}/g day 7 bacterial counts. Student-Newman-Keuls test after one-way ANOVA was performed (Graphpad Prism® Software), and differences were deemed to be statistically significant if P ≤ 0.05.

In vivo bacterial counts are summarized in table 1. Vancomycin constant infusion (VIV) did not demonstrate significant antibacterial activity in any of the three compartments (JF, BM, and BO) tested over the 4-day treatment. CDA unloaded showed no intrinsic activity or inhibition of the development of osteomyelitis, even in addition to a constant infusion of vancomycin. No difference was found with the reference group. CDA loaded with 25 µg/mg of vancomycin (CDA25), without combination of systemic vancomycin, did not show sufficient activity. However, CDA loaded with 25 µg/mg of vancomycin, in addition to constant infusion, was active in bone and bone marrow, with -2.40 log_{10} and -3.14 log_{10} (P<0.05), respectively. CDA loaded with increased concentration of vancomycin (100 µg/mg), without systemic infusion, showed significant antimicrobial activity in bone and marrow, but not in joint fluid. The addition of a constant infusion of vancomycin to CDA100 showed the best results in bone (- 4.63 log_{10}) and bone marrow (- 4.03 log_{10}). CDA loaded with 50 percent of vancomycin showed similar results as the CDA100 in bone marrow but not in bone, even with the addition of constant infusion. The bacterial counts in the joint fluid showed a linear response with loading, with the exception of the CDA500+V group. The impact on the bacterial counts of the constant infusion is summarized in figure 1.

The acute osteomyelitis model provides a rapid assessment of antibiotic activity as compared with the chronic model (12). This study showed that vancomycin constant intra-vascular infusion was not able to decrease bacterial counts after a four-day treatment. CDA unloaded alone or in concert with vancomycin infusion demonstrated no antimicrobiological activity.
Low concentrations of antibiotic (25µg/mg) loaded into the CDA, were deemed efficient at treating the bone and marrow when in combination with systemic infusion. 50 µg/mg of vancomycin incorporated via wet granulation was the maximum level usable in clinical practice, CDA matrix showing limits to drug incorporation. This was determined as the highest level of loading that did not compromise the osteoconductive properties of this artificial mineral compound. Nevertheless, the greatest reduction in bacterial counts in situ in bone and bone marrow was obtained with lower concentrations (CDA100) in addition to constant infusion of vancomycin. Increased concentration of incorporated vancomycin (500 µg/mg) did not show significant enhancement. The mortality rate, as an indirect indicator of the efficiency of the treatment, confirms this data. Bone infections are very difficult to treat, and localized treatments in addition to systemic approaches are becoming more relevant as a means to limit the bacterial invasion and improve the prognosis of the osteomyelitis. CDA loaded with 100 µg/mg of vancomycin may be the most effective antibiotic concentration to avoid adverse effects and toxicity of vancomycin.
References


TABLE 1. Difference between day 7 and day 3 of bacterial counts in joint fluid (JF), bone marrow (BM), and bone (BO) and relationship to mortality rates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Mean ± SD ∆log_{10} CFU/g of tissue (day 7 – day 3)</th>
<th>Mortality rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JF</td>
<td>BM</td>
</tr>
<tr>
<td>VIV</td>
<td>6</td>
<td>-0.03 ± 0.93</td>
<td>-0.73 ± 1.84</td>
</tr>
<tr>
<td>CDA</td>
<td>9</td>
<td>-0.49 ± 0.32</td>
<td>0.56 ± 0.31</td>
</tr>
<tr>
<td>CDA+V</td>
<td>6</td>
<td>0.30 ± 0.43</td>
<td>0.75 ± 0.35</td>
</tr>
<tr>
<td>CDA25</td>
<td>7</td>
<td>-0.56 ± 0.95</td>
<td>-1.89 ± 1.29</td>
</tr>
<tr>
<td>CDA25+V</td>
<td>5</td>
<td>0.68 ± 1.32</td>
<td>-3.14 ± 0.86*</td>
</tr>
<tr>
<td>CDA100</td>
<td>9</td>
<td>-0.05 ± 1.03</td>
<td>-3.41 ± 1.43</td>
</tr>
<tr>
<td>CDA100+V</td>
<td>7</td>
<td>-0.90 ± 0.39</td>
<td>-4.03 ± 1.33*</td>
</tr>
<tr>
<td>CDA500</td>
<td>7</td>
<td>-0.29 ± 1.16</td>
<td>-4.08 ± 1.27*</td>
</tr>
<tr>
<td>CDA500+V</td>
<td>11</td>
<td>-1.69 ± 0.62*</td>
<td>-4.21 ± 1.16*</td>
</tr>
</tbody>
</table>

n: number of animals

*P<0.01 vs VIV, CDA and CDA+V

*= P<0.05 vs VIV, CDA and CDA+V

<sup>1</sup> = P<0.05 vs CDA25+V, CDA100, CDA500+V and CDA500
FIG. 1. Effect of vancomycin constant infusion (black forms) on bacterial counts in joint fluid (diamonds), bone marrow (circles) and bone (triangles) for CDA25, CDA100 and CDA500.