Effect of Local Delivery of Vancomycin and Tobramycin on Bone Regeneration

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Objective: A bone defect rat model was established to investigate the osteogenic effect of local delivery two antibiotics (vancomycin and tobramycin powder) on bone regeneration.

Methods: Twenty-four Sprague–Dawley (SD) male rats (6 to 8 weeks, 200 to 250 g) were used in this study. All these rats were randomly divided into four groups. Based on dose conversion between rat and human via body surface area, the rat dose of two antibiotics was 88 μg/g and 176 μg/g for vancomycin and tobramycin, respectively. Con group (no antibiotic), Van group (vancomycin, 88 μg/g), Tob group (tobramycin 176 μg/g), and Van + Tob group (vancomycin 88 μg/g combined with tobramycin 176 μg/g). A 5.0-mm full-thickness standardized mandibular bone defect was performed with a drill in each rat and different antibiotic powders were placed over the bone defect space, respectively. All these animals were sacrificed after 12 weeks post-operation. The mandible bones were harvested for further radiographic and histologic analysis. The bone volume/total volume (BV/TV) ratio, bone volume (BV), and bone fractional area (BFA) in the defect area via micro-computed tomography (μCT scanning) were further analyzed. Then, we performed a histological assessment via hematoxylin and eosin (H&E) and Masson’s trichrome staining to analyze bone regeneration and also analyze the number of osteoblasts per field.

Results: There were no postoperative deaths, signs of vancomycin-related or tobramycin-related toxicity, or signs of systemic illness in any of the four groups. All wounds healed well, and no complications or surgical site infection were observed in all rats. From the μCT scans analyses, there was less bone regeneration in the Van group than in the Con group (BV/TV: F = 64.29, R² = 0.9602; P = 0.0052; BFA: F = 76.17, R² = 0.9662, P = 0.0007; BV: F = 194.4, R² = 0.9865, P = 0.0022). However, when the tobramycin and vancomycin were combined, an increase in bone defect re-ossification was found in the Van+Tob group than in the Van group (BV/TV: F = 64.29, R² = 0.9602, P = 0.0033; BFA: F = 76.17, R² = 0.9662, P = 0.0006; BV: F = 194.4, R² = 0.9865, P = 0.0033). Routine H&E and Masson staining supported the finding of μCT scanning. Quantitative indices confirmed that both the bone regeneration and the number of osteoblasts per field in the defect area was higher in the Van+Tob group than in the Van group (percentage of bone tissue: F = 145.7, R² = 0.9562, P = 0.0008; number of osteoblasts per file: F = 67.3, R² = 0.9098, P < 0.0001). There was no significant difference between the Con group and the Van+Tob group on the number of osteoblasts each field (F = 145.7, R² = 0.9562, P > 0.9999).

Conclusion: For bone defect, local application of vancomycin combined with tobramycin was recommended over vancomycin alone. This animal study presents data suggesting that the use of local delivery of vancomycin and tobramycin should be investigated further in clinical studies.

Key words: Bone regeneration; Local delivery; Osteogenesis; Tobramycin; Vancomycin
Introduction

Traditional systemic intravenous prophylactic antibiotics could reduce the infection rates and has become a standard preoperative strategy. However, the surgical site infection is a common complication in orthopedic surgery that can increase the cost of treatment. Furthermore, the offending organisms are becoming more resistant to antibiotics. Studies showed that more than 50% of Staphylococcus isolates from postoperative infections are insensitive to ceph- alosporins. Traditional systemic intravenous prophylactic antibiotics mainly rely on the diffusion into the surgical site. Although the adequate serum antibiotics concentrations, the concentration of antibiotic scan are lower than the minimum inhibitory concentration of the target tissue. However, local application of antibiotics to the surgical sites could keep an adequate therapeutic drug level, providing an effective minimum inhibitory concentration in the surgical sites.

Local administration of antibiotics powder has been advocated as an adjuvant measure to help prevent surgical site infection. Local intrawound delivery of antibiotic powder after orthopedic surgery has been shown to reduce infection rates as much as 10-fold. This prophylactic method is better than intravenous and oral antibiotics, because of the adverse effects of systemic administration of antibiotics such as nephrotoxicity. Furthermore, local delivery can reduce antibiotic resistance. Thus, topical delivery antibiotic powder has become an increasing measure for reducing the postsurgical infection rate in orthopedic procedures. Recent studies have suggested that vancomycin or tobramycin is safe and could reduce postoperative infection rates.

Vancomycin is an antibiotic used to treat a number of bacterial infections, such as bone and joint infections caused by methicillin-resistant Staphylococcus aureus (MRSA). Topical delivery of vancomycin in the surgical site is not correlated with vancomycin-resistant bacteria. However, topical administration of vancomycin powder can reduce the infection of gram-positive bacteria, but not the gram-negative bacterial infections. There was an increased incidence of gram-negative organisms in infections after local application of vancomycin in the wound versus no vancomycin.

Tobramycin belongs to the aminoglycoside family and is derived from Streptomyces tenebrarius. Tobramycin is widely used in the treatment of moderate to severe bacterial infections due to sensitive organisms. Tobramycin transports into the cell and interferes with the initiation complex between messenger RNA and the 30S subunit, thereby inhibiting initiation of protein synthesis, consequently leading to bacterial cell death. It has a narrow spectrum and is used to treat various types of gram-negative bacteria infection. It is especially effective against species of Pseudomonas aeruginosa. Vrabec et al. evaluated the local concentration of tobramycin in an in vivo study and found that local tobramycin bone cement can be used to obtain short-term suprathapeutic local concentrations in the surgical site while maintaining minimum serum tobramycin levels. A recent study proved that the topical application of tobramycin combined with vancomycin powder further reduced the infection rate than vancomycin alone.

Another study showed that local delivery of vancomycin and tobramycin could effectively inhibit the acute prosthetic joint infection. Because they have found that all bacteria in their study resistant to tobramycin were susceptible to vancomycin and vice versa. Thus, the combination of topically used of vancomycin and tobramycin powder provide a broad spectrum of antimicrobial activity.

However, studies have suggested that local delivery of vancomycin inhibits the osteogenesis process. For example, Eder et al. used primary osteoblast cells to perform an in vitro study, and proved that intrawound application of high dosage vancomycin interferes with bone regeneration and increases the risk of non-union. Seavey et al. also demonstrated that the heterotopic ossification formation could be reduced by topical application of vancomycin powder. Furthermore, Ishida et al. performed lumbar fusion surgery on 60 Lewis rats using syngeneic iliac crest allograft mixed with clinical bone-graft substitute and two different antibiotics. They found that local application of vancomycin detriment the fusion-mass formation in a rat model. They also found that tobramycin administration could augment fusion-mass formation. Nart et al. assessed the clinical and radiographic outcomes of the regenerative treatment of peri-implantitis using a vancomycin and tobramycin impregnated allograft after a 12-month period. They found that the use of locally delivered antibiotics together with the bone graft may reduce the undesirable effects related to the systemic administration and the risk of resistances and the application of combined antibiotics with collagen membrane yielded positive outcomes of radiographic bone fill. Thus, the combination of vancomycin and tobramycin antibiotics covers the majority of the relevant spectrum in orthopedic surgery. And the addition of tobramycin may rescue the osteogenic potential inhibited by vancomycin in vivo.

In this study, we would like: (i) to examine the osteogenic effects of topical delivery of vancomycin; (ii) to evaluate the combination of vancomycin and tobramycin on osteogenic effects in a bone defect model; and (iii) to discuss the safety of local application vancomycin and tobramycin powder. We first established a mandibular bone defect rat model in vivo. Then the vancomycin and/or tobramycin were topical administered in the surgical site. Twelve weeks post operation, the samples were collected. We further evaluated the osteogenic effects of topical delivery of dural antibiotics via radiographic and histological analysis.

Materials and Methods

Animal Care and Surgical Procedures

All animal experiments were approved by the protocol of the Institutional Animal Care and Use Committee at China Medical University (CMU). Sprague-Dawley (SD) male rats (n = 24, 6 to 8 weeks, 200 to 250g) were provided by CMU. We used clinical concentration of antibiotics in this study
and revealed significant new bone formation within the defects. Masson’s trichrome is a three-color staining protocol used for distinguishing cells from surrounding connective tissue. Most recipes produce red keratin and muscle fibers, blue or green collagen and bone, light red or pink cytoplasm. The results were observed under a microscope and analyzed by ImageJ software.

Outcome Measurements

Bone Volume (BV)
BV is the mineralized bone within the defect volume. It was used to evaluate the new bone formation volume in the total defect volume. The total bone defect volume was 19.625 mm³. The maximum volume of BV was 19.6 mm³, so BV < 1.96 mm³ is poor, 1.97–4.9 mm³ is fair, 5.0–9.8 mm³ is good, and 9.9–19.6 mm³ is excellent. The BV was compared among the four groups.

Bone Volume/Total Volume Ratio (BV/TV)
BV/TV stands for bone volume over total volume. In other words, BV/TV is the volume of mineralized bone per unit volume of the sample. It indicates the fraction of a given volume of interest (VOI, the TV) that is occupied by mineralized bone (BV). BV/TV calculated: new bone volume (mm³)/total bone defect volume (mm³) × 100%. 0%–10% is considered poor, 10%–25% is fair, 25%–50% is good, and 50%–100% excellent.

Bone Fractional Area (BFA)
BFA is the percentage of new bone formation area within the total bone defect area in a 2D plane. BFA was also performed to determine the area within the defect covered by new bone formation. BFA calculated: new bone area (mm²)/total bone defect area (mm²) × 100%. 0%–10% is considered poor, 10%–25% is fair, 25%–50% is good, and 50%–100% excellent.

The Number of Osteoblasts per field
The number of osteoblasts per field indicates the osteogenesis of the bone defect. It was used to evaluate the new bone formation volume indecent volume. It is the number of cells in a given volume of interest (VOI) of new bone area. The number of cells was compared among the four groups.

The Percentage of Bone Tissue (Bone Tissue %)
The percentage of bone tissue stands for mineralized bone area over a defect area. It indicates the bone defect re-ossification. It was used to evaluate the new bone formation area in defect area. Bone Tissue percentage calculated as new bone formation (mm³)/volume of interest (VOI) (mm³) × 100%. 0%–10% is considered poor, 10%–25% is fair, 25%–50% is good, and 50%–100% excellent.

Formula 1: The rat dose translated from human dose based on BSA.

$$\text{Ratdose} = \text{Humandose} \times \frac{\text{humanKm}}{\text{ratKm}}.$$
**Statistical Analysis**

All statistical analyses were performed by Prism software 8.2 (GraphPad) for macOS. One-way analysis of variance was used to compare the differences of BV, BV/TV, BFA, number of osteoblasts per field and the percentage of bone tissue. All the results were biologically replicated three times. All reported $P$ values are 2-sided, and values of $P < 0.05$ were considered as statistically significant.

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**Fig. 1** Antibiotics affect bone regeneration in an animal model was evaluated by μCT scans at 12 weeks. The osteogenic effect of *in vivo* experiments from four different groups: Con group (A and B), Van group (C and D), Tob group (E and F), and Van+Tob group (G and H) (Scale bar, 2 mm). And comparisons of μCT bone defect fusion score among four groups: the bone volume/total volume ratio (BV/TV) (I), bone formation area (BFA) (J), and bone volume (BV) (K) in the defect area was calculated. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. (Con: control; Van: Vancomycin; Tob: tobramycin).
Results

**Bone Regeneration on μCT Scans**

Twelve-weeks post-operation, all wounds were healed well, and no complications were observed in all rats. After sacrifice, the new bone formation was evaluated by μCT. The representative samples of 3-dimensional μCT scans in each group are shown in Fig. 1–H. We found there was less bone regeneration in the Van group than in the Con group (BV/TV: F = 64.29, R² = 0.9602, P = 0.0052; BFA: F = 76.17, R² = 0.9662, P = 0.0007; BV: F = 194.4, R² = 0.9865, P = 0.0022.) (Fig. 1C and D). When the tobramycin and vancomycin were combined, an increase in bone defect re-ossification was found in the Van+Tob group (Fig. 1G and H). Quantitative indices confirmed that bone formation in the defect area was higher in the Van+Tob group than in the Van group (BV/TV: F = 64.29, R² = 0.9602, P = 0.0033; BFA: F = 76.17, R² = 0.9662, P = 0.0006; BV: F = 194.4, R² = 0.9865, P = 0.0033) (Fig. 1I–K).

**Bone Tissue and the Number of Osteoblasts on Histologic Analysis**

Routine H&E and Masson staining (Fig. 2) supported the finding of μCT scanning. The results showed that less bone formation area was observed in the Van group (Fig. 2C and D) (F = 145.7, R² = 0.9562; Van vs. Con P < 0.0001; Van vs. Tob P < 0.0001; Van vs. Van+Tob P = 0.0008); The area of bone formation in the Tob group (Fig. 2E and F) was higher than that in the Con group (Fig. 2A and B) (F = 145.7, R² = 0.9562, P < 0.0001), and better bone regeneration was observed in the Van+Tob group (Fig. 2G and H) than that in the Van group (Van vs. Van+Tob F = 145.7, R² = 0.9562, P = 0.0008). The number of osteoblasts each field (×200) of the Tob group was higher than that of the Con group (F = 67.3, R² = 0.9098, P < 0.0001) and the number of osteoblasts per field of the Van group was the lowest among the four groups (F = 67.3, R² = 0.9098, Van vs. Con P < 0.0001; Van vs. Tob P < 0.0001; Van vs. Van+Tob P < 0.0001). There was no significant difference between the Con group and the A

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**Fig. 2** Histological analysis of bone regeneration of four groups in bone defect model at 12 weeks. Representative images (Scale bar 200 μm) of H&E staining and Masson trichrome staining in the Con group (A and B), Van group (C and D), Tob group (E and F), and Van+Tob group (G and H), indicating the new bone formation inside the dashed line. The number of osteoblasts (I) and the percentage of bone tissue (J) were compared among the four groups. ***P < 0.001, ****P < 0.0001. (Con: control; Van: Vancomycin; Tob: Tobramycin).
Van+Tob group ($F = 67.3$, $R^2 = 0.9098$, $P > 0.9999$) (Fig. 2I). There were no signs of surgical site infection in all the samples.

**Discussion**

In this study, we have demonstrated that local delivery of vancomycin may have detrimental effects on bone regeneration in the bone defect area, as evidenced by radiographic and histological analysis. However, tobramycin can promote bone regeneration. Additionally, the combination of vancomycin-tobramycin had no inhibitory effect on bone regeneration. We have proved the osteogenic effect of intraoperative administration of these two antibiotics on bone defects in an animal model.

**Vancomycin Inhibits Osteogenesis**

Local delivery of vancomycin and/or tobramycin therapy is an effective and safe method to reduce bacterial infections in orthopedic surgery. They have been widely used for bone defects, open fractures and bone tissue infections. However, a higher concentration of vancomycin influences the pH of the cell culture medium, which reduces the formation of osteoblast pseudopodia. Furthermore, the higher concentration of vancomycin suppresses osteoblast migration, inhibits the cell proliferation and viability of osteoblast. Our results proved that vancomycin can also reduce bone regeneration in vivo. Another in vivo study showed that local delivery of vancomycin powder affects the bone fusion process. In contrast, another study demonstrated that bone morphogenetic protein-2 (BMP-2) combined with vancomycin did not affect osteogenesis in vivo. However, BMP-2 addition may result in heterotopic ossification in orthopedic surgeries and offset the negative effect of vancomycin on bone regeneration.

**Tobramycin Promote Osteogenesis**

The addition of tobramycin has a synergistic-like effect on bactericidal activity of vancomycin. Several studies recommended topical administration of vancomycin combined with tobramycin powder intrawound. Recently, it has been proved that tobramycin promotes fracture healing by accelerating osteogenesis differentiation of mesenchymal stem cells through activating the Wnt/β-catenin pathway. Ishida et al. also found that tobramycin appeared to augment bone fusion in a rat model, which is comparable with our findings. Another comparative study of tobramycin and vancomycin showed there were better osteogenesis scores in the tobramycin impregnated material group. Additionally,
a study has proved that local delivery of tobramycin had no cytotoxic and did not affect cell number of osteoblasts\textsuperscript{32}. Thus, tobramycin powder could promote bone formation and counteract the negative effect of vancomycin on osteogenesis (Fig. 3).

**Limitations**

There are several limitations in this study. First, we only explored the osteogenesis of two antibiotics (vancomycin and tobramycin) in our study, other antibiotics such as gentamicin\textsuperscript{33}, ampicillin\textsuperscript{34}, and amikacin\textsuperscript{35} need to be further explored. Second, we only studied the clinical concentration of these antibiotics. However, we use the BSA as a factor to convert the dose of antibiotics for translation from humans to rats, which better simulate the human equivalent doses in a rat model. We need to further find out the proper concentration and ratio of vancomycin to tobramycin and recommended doses for patients. Finally, we did not address bacterial infection in our animal models. We would like to investigate this in our future in vivo studies.

**Conclusion**

In conclusion, topical delivery of vancomycin can reduce osteogenesis in the bone defect area. However, tobramycin could promote bone regeneration, and further offset the negative osteogenic effect caused by vancomycin. And we recommend the local application of vancomycin combined with tobramycin for anti-infection of bone defect surgery. The safety and efficacy local delivery of vancomycin-tobramycinin reducing the risk of both non-union and surgical site infection in orthopedic surgery needs further exam via prospective clinical trials.

**Data availability statement**

All data included in this study are available upon request by contact with the corresponding author.

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**Author Contributions**

C: Conceptualization, Supervision, Validation: J.W.; Funding acquisition, Project Administration: W.H.; Methodology, Investigation: L.Z.; Formal Analyses and Data Curation: L.Y.
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