**TITLE:**

Treatment with Vancomycin Loaded Calcium Sulphate and Autogenous Bone in an Improved Rabbit Model of Bone Infection

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**KEYWORDS:**

Bone infection, rabbit model, *Staphylococcus aureus*, tibia, vancomycin loaded calcium sulphate, autogenous bone

**SUMMARY:**

This study presents an improved rabbit model infected with *Staphylococcus aureus* by blocking the same amount of bacteria in bone marrow. Vancomycin loaded calcium sulphate and autogenous bone are used for antibiotic and bone repair treatment. The protocol could be helpful for studying bone infection and regeneration.

**ABSTRACT:**

Bone infection results from bacterial invasion, which is extremely difficult to treat in clinical, orthopedic, and traumatic surgery. The bone infection may result in sustained inflammation, osteomyelitis, and eventual bone non-union. Establishment of a feasible, reproducible animal model is important to bone infection research and antibiotic treatment. As an in vivo model, the rabbit model is widely used in bone infection research. However, previous studies on rabbit bone infection models showed that the infection status was inconsistent, as the amount of bacteria was variable. This study presents an improved surgical method for inducing bone infection on a rabbit, by blocking the bacteria in the bone marrow. Then, multi-level evaluations can be carried out to verify the modelling method.

In general, debriding necrotic tissue and implantation of vancomycin-loaded calcium sulphate (VCS) are predominant in antibiotic treatment. Although calcium sulphate in VCS benefits osteocyte crawling and new bone growth, massive bone defects occur after debriding. Autogenous bone (AB) is an appealing strategy to overcome bone defects for the treatment of massive bone defects after debriding necrotic bone.

In this study, we used the tail bone as an autogenous bone implanted in the bone defect. Bone repair was measured using micro-computed-tomography (micro-CT) and histological analysis after animal sacrifice. As a result, in the VCS group, bone non-union was consistently obtained. In contrast, the bone defect areas in the VCS-AB group were decreased significantly. The present modeling method described a reproducible, feasible, stable method to prepare a bone infection model. The VCS-AB treatment resulted in lower bone non-union rates after antibiotic treatment. The improved bone infection model and the combination treatment of VCS and autogenous bone could be helpful in studying the underlying mechanisms in bone infection and bone regeneration pertinent to traumatology orthopedic applications.

**INTRODUCTION:**

Bone infection usually results from bacteria or other microorganism invasion after trauma, bone fracture, or other bone diseases1. Bone infection may induce a high level of inflammation and bone tissue destruction. In the clinic, *Staphylococcus aureus* (*S. aureus*) is the predominant causative agent of bone infection2,3. The bone infection is painful, debilitating, and often takes a chronic course that is extremely difficult to treat4. At present, debridement of necrotic tissue and implanting of vancomycin-loaded calcium (VCS) beads have been confirmed as an efficient strategy for controlling local infection5,6. However, 10% to 15% of patients experienced a prolonged bone repair process, delayed union, or non-union after anti-infection treatment7. The large segment of a bone defect is the most difficult issue for orthopedic surgeons. An autologous bone graft is considered the optimal bone replacement in bone non-union treatment8,9.

To date, most of the studies on bone infection and autologous bone implantation have been conducted in various kinds of animal models, such as rats, rabbits, dogs, pigs and sheep10,11. Rabbit models are most commonly used for bone infection studies, as first performed by Norden and Kennedy in 197012,13. In our previous study, we used rabbit models following Norden's method, and we found that the quantity of *S. aureus* injected into bone marrow could not be quantified accurately, as the blood leaking out of bone marrow led to bacteria solution overflow.

This article presents an improved surgical method for inducing bone infection on rabbits. At the end of the procedure, a blood biochemistry test, a bacteriological examination, and a histopathologic examination were performed to verify the bone infection model. Then, VCS was implanted to inhibit infection, and autogenous bone was implanted to promote bone regeneration.

**PROTOCOL:**

The rabbits used in the present study were treated in accordance with the Guide for the Care and Use of Laboratory Animals. All the experimental procedures were followed by the rules of the Bioethics Committee of Zhejiang Academy of Traditional Chinese Medicine.

1. **Preparation of the** **bacterial suspension**
   1. Dissolve 0.5 mg of *S. aureus* freeze-drying powder (ATCC 6538) with 0.3 mL of Luria-Bertani culture medium. Mix suspension completely.
   2. Streak the bacteria suspension onto tryptic soy agar plates and incubate the bacterial colonies at 37 °C for 16 h.
   3. Select a single bacterial colony forming unit (CFU) and culture in tryptic soy broth tubes for 24 h. Perform a subculture for approximately 24 h at 37 °C, and obtain mid-logarithmic growth phase bacteria after 16 to 18 h, when the optical density (OD) value is 0.6 at 600 nm14.
   4. Transfer 1 mL of bacteria suspension into a centrifuge tube. Centrifuge for 5 min at 825 x *g* and 4 °C, and discard the supernatant. Resuspend and wash the bacteria with 100 µL of phosphate buffered saline (PBS); repeat this step 3 times. Finally, resuspend bacteria with 3 mL of PBS.
   5. Estimate the bacteria concentration using McFarland's turbidimetry15.
      1. Transfer 100 to 500 µL of bacteria suspension to a colorimetric tube until the turbidity is equivalent to a 0.5 McFarland standard.
      2. Assess turbidity by visual comparison to the 0.5 McFarland, when the content of bacteria reaches approximately 108 CFU/mL.

NOTE: Make sure the volume of bacteria suspension is sufficient for the following protocols. For every rabbit, the volume of bacteria suspension is less than 1 mL.

* 1. Transfer 0.2 mL of bacterial suspension to an agar plate and apply it evenly. Incubate the plate at 37°C for 16 h. Count the bacteria colonies to verify the CFU of bacteria suspension.

1. **Preparation of** **bone infection models**
   1. Keep male New Zealand white rabbits, aged 3 months, in individual cages, under air-controlled conditions (20 ± 1 °C) and 12 h/12 h light-dark illumination cycles. Offer routine diet and tap water daily.
   2. Ensure that at the time of surgery that the rabbit weighs more than 3 kg.
   3. Anaesthetize rabbits by intraperitoneal injection with pentobarbital sodium (3 mg per 100 g of body weight). Make sure rabbits are fully anesthetized by a failure to respond to a paw pinch. Fix rabbits on the operating table during operation procedure.

NOTE: Make sure that the modeling procedure duration is less than 1 h.

* 1. Shave the proximal tibia region using an electric shaver against the direction of hair growth. Disinfect the skin by applying a povidone-iodine solution.
  2. Mark the upper end of the tibia and the drilling hole position for injection with *S. aureus* (the distance to the upper end of the tibia is 1.5 cm) with pen and ruler. Make sure the drilling hole positions are in the middle of the tibial plateau horizontally (**Figure 1A**).

* 1. Cut tibia skin using a No. 11 scalpel and make a 1 cm incision in the periosteum (**Figure 1B,C**). Punch a 2 mm diameter hole in the tibia using an electric bone drill unit (**Figure 1D**).
  2. Press the 2 mm diameter holes in the tibial plateau with a cylinder of bone wax of 2 mm diameter and 2 mm height (**Figure 1E**). Remove the spare bone wax along the horizontal plane of the tibial plateau (**Figure 1F**). Check that the 2 mm hole is full of bone wax (**Figure 1G**).

NOTE: Ensure that the holes are full of bone wax by checking the hole with or without blood overflow.

* 1. Sew up the periosteum and skin with absorbable surgical suture in a vertical mattress suture to prevent the animal from chewing the sutures (**Figure 1H**).
  2. Inject 1 x 108 CFU/mL of *S. aureus* solutions (30 µL per 100 g of body weight) with a 1 mL asepsis injector (**Figure 1I**). Make sure the needles penetrate the bone wax and inject the *S. aureus* solution into bone marrow slowly.

* 1. Keep the animal in warm and clean conditions to avoid heat loss after modelling. Monitor respiratory rate and heart rate. After waking up, house the rabbits in individual cages with free access to food and water.

1. **Evaluation of bone infection model**
   1. At days 7, 14, 21 and 28after infection, place rabbits into the rabbit fixer with the head and ear outside of the fixer.
   2. Draw 2 mL of blood from the auricular veins into a dipotassium ethylenediaminetetraacetic acid (EDTA-K2) anticoagulant blood container. Draw 1 mL of blood from a blood vessel into a blood container. Centrifuge the serum for 10 min with a speed of 651 x *g* at room temperature. 
      1. Determine white blood cell count (WBC) in whole blood using a blood biochemical analyzer, and C-reactive protein (CRP) by an enzyme-linked immunosorbent assay (ELISA) method16.
   3. At days 7, 14, 21 and 28 after infection, anaesthetize one model rabbit with pentobarbital sodium at the dosage of 3 mg per 100 g body weight. Cut tibia skin using a No. 11 scalpel and make a 2 cm incision in the periosteum (**Figure 2A**).
   4. Clean bone wax. Debride necrotic bone by punching two adjacent 4.8 mm diameter holes using an electric bone drill unit (**Figure 2B**). Debride necrotic bone marrow and granulation tissue using a bone spoon (**Figure 2C**).

NOTE: Clean bone tissue during the debridement to avoid bone tissue remaining in the bone marrow.

* 1. Scrape and clean the bone tissue between the two holes (**Figure 2D**).

* 1. Spread 1 mL of bone marrow onto sheep blood agar plates. Incubate plates overnight at 37 °C. Select plates of 30-300 colonies, and calculate the number of colonies.

* 1. At the end of day 28 after infection, extract tibia specimens along the edges of knee and ankle joints. Fix the tibia specimens in 4% paraformaldehyde for 24 h. Decalcify the tibia specimens in 10% EDTA for 8 weeks.
  2. Dehydrate the tibia specimens in a graded series of ethanol dilutions, and then embed in paraffin wax. Cut 4 consecutive 5 µm sections from the coronal planes. Stain sections with a hematoxylin and eosin (H&E) staining kit.
  3. Use a microscope to view the stained sections and record transmitted light images with standard software.

1. **Preparation of** **VCS beads**

* 1. Add 1 g of vancomycin hydrochloride powder to 9.5 g of medical grade calcium sulphate, and then add 3 mL of normal saline to the mixed power. Mix them thoroughly with a spatula for 30 to 45 s.
  2. Place the mixed product into a flexible silica gel mold (cylinder of 4.8 mm diameter and 4.8 mm height), and dry at room temperature for 15 min. Remove the VCS beads by flexing the mold.

1. **Antibiotic treatment and implantation of** **autogenous bone**
   1. Anaesthetize model rabbits with pentobarbital sodium at the dosage of 3 mg per 100 g body weight on the 28th day after infection. Shave the proximal tibia region using an electric shaver. Disinfect the skin by applying povidone-iodine solution.

NOTE: Make sure that the modelling procedure is less than 1 h.

* 1. Shave the tail region using an electric shaver and disinfect the tail by applying povidone-iodine solution.
  2. Cut down the tail using surgical scissors. Cut tail skin using a No. 11 scalpel and reveal the tail bone. Remove any muscle, soft tissue and periosteum.
  3. Detach the tail bone at each joint and transfer the bone fragment to a 100 mm plastic dish containing sterile saline.
  4. Sew up the skin at the tail region with absorbable surgical sutures in a vertical mattress suture to prevent the animal from chewing the sutures.
  5. Implant 4 pieces of VCS beads (cylinder of 4.8 mm diameter and 4.8 mm height, 1.25 mg vancomycin per piece of bead) into the marrow cavity using curved tweezers (**Figure 2E**).
  6. Fill the bone defect with 8 pieces of autogenous bones (cylinder of 2 mm diameter and 4 mm height per each piece) using curved tweezers (**Figure 2E**).

* 1. Sew up the periosteum and skin with absorbable surgical sutures in a mattress suture manner (**Figure 2F**).

NOTE: Keep the temperature at 25 °C during the surgery.

* 1. Keep the animal in warm and clean conditions to avoid heat loss after surgery. Monitor respiratory rate and heart rate. After waking up, house the rabbits in individual cages with free access to food and water.

1. **Ass****essments of antibiotic activity**
   1. Put rabbits into a rabbit fixer, and place the head and ear outside of the fixer at 2, 4, 6 and 8 weeks after treatment.

* 1. Draw blood from auricular veins with EDTA-K2 anticoagulant blood vessel. Draw 1 mL of blood from a blood vessel into a blood container. Centrifuge the serum for 10 min with a speed of 651 x *g* at room temperature.
  2. Determine the white blood cell count (WBC) in whole blood by using blood biochemical analyzer, and C-reactive protein (CRP) by an ELISA method16.

1. **Assessments of** **bone regeneration**
   1. Euthanize rabbits by injecting with an over dosages of pentobarbital sodium, at the end of 8 or 12 weeks after treatment.
   2. Extract tibia specimens, along the edges of knee and ankle joints. Debride muscles and fascial layers.
   3. Analyze structure of tibia by using micro-computed tomography (micro-CT). Choose an oval area 4.8 mm diameter and 9.6 mm long as the region of interest (ROI). Reconstruct 3D model images using bitmap data.
   4. Choose scores of the ratio of bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecula number (Tb.N) and trabecular separation (Tb.Sp)from the3D models to assess bone regeneration.

**REPRESENTATIVE RESULTS:**

**Evaluation of Bone Infection Model**

After infection with *S. aureus*, the pathological manifestations of rabbits were similar to the representative symptom of chronic osteomyelitis in the clinic. In our study, 30 rabbits were infected, and subjected as a model group, and 10 rabbits were subjected as control animals. All the model rabbits have infected sinuses of the tibia local site, with white and yellow pus over flow from the sinuses (**Figure 3A**). The results of H&E staining indicate that bacterial aggregates are located around dead bone in the model group, and normal osteocytes cannot be identified. The levels of CRP and WBC are higher in the model group than the control group (**Figure 3B**). The necrotic bone marrow lysates are streaked on agar plates, which result in an increased number of colonies for the model group after infection (**Figure 3C**). At the end of modelling, there were 3 rabbits dead because of serious infection. The remaining infected rabbits were identified as the bone infection model and were divided into 3 groups: model group, VCS group, and VCS-AB group.

**Assessments of Antibiotic Activity and Bone Regeneration**

At 2, 4, 6, and 8 weeks after treatment with VCS or VCS-AB, the levels of CRP and WBC are reduced significantly (**Figure 4A**). After 12 weeks of implantation of VCS and bone allograft, tibial defects of the VCS-AB group seemed fully coalescent. Tibial plateau surfaces of the VCS-AB group are flatter than that of the VCS group (**Figure 4B**). 2D reconstruction images indicate a progressive increase in bone volume during the 12 week period after treatment with VCS-AB and VCS, while bone loss is significant in the model group. To analyze the bone regeneration quantitative indexes, an oval area 4.8 mm diameter and 9.6 mm long was chosen as the region of interest (ROI) **(Figure 4C**), and 3D model images were reconstructed using bitmap data. The micro-CT indexes BV/TV in the model group were significantly lower than that in the VCS and VCS-AB groups. The Tb.N and Tb.Th scores in the VCS-AB group were significantly higher than those in the model and VCS group. Moreover, the Tb.Sp scores in the VCS-AB group are markedly lower than that in the model group and VCS group (**Figure 4D**).

These results suggest that infection with *S. aureus* causes increasing WBC and CRP in the model group, which can be decreased using the VCS. The implantation of VCS is regarded as the optimal antibiotic treatment. However, the bone defect is observable in the VCS group. The VCS and autogenous bone treatment increase the trabeculae thickness and trabeculae number and decrease the trabecular separation. The VCS-AB treatment showed capability of promoting bone healing.

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Surgical preparation of the bone infection model.**(**A**) Shows the punching position on the tibia. The distance between the drilling hole position for injection with *S. aureus* to the upper end of tibia is 1.5 cm. (**B**) Incision made in the skin to expose the periosteum. (**C**) Shows incision made through the periosteum to expose the tibia. (**D**) Punch a 2-mm diameter hole in the tibia. (**E**) Fill the 2 mm diameter hole full of bone wax. (**F**) Cut off spare bone wax. (**G**) Show the bone wax filling the bone defect. (**H**) Sew up the periosteum and skin. (**I**) Inject with *S. aureus* solution.

**Figure 2. Preparation of** **bone allograft and antibiotic treatment.** (**A**) Incision made in the skin to expose periosteum. (**B**) Punch two adjacent 4 mm diameter holes. (**C**) Debride necrotic bone and inflammatory bone marrow. (**D**) Scrape and clean the bone tissue between the two holes to make a long circle of 4 mm diameter and 8 mm long. (**E**) Fill the hole with VCS and bone allograft. (**F**) Sew up the periosteum and skin.

**Figure 3. Evaluation of bone infection model.** (**A**) Appearance features of the rabbit legs infected with *S. aureus*, and typical histopathology images of the rabbit tibia in the model and control groups. Blue arrow: osteocyte; pink arrow: bone trabeculae; yellow arrow: bacterial aggregates; green arrow: dead bone. (**B**) The WBC and CRP results in the rabbit serum at the time points of before modelling, 7, 14, 21 and 28 days after infection. The columns represent the mean ±SE, \**p* < 0.05 *vs.* the control group. (**C**) The number of bacterial colonies in the tibia marrow counted following overnight incubation. The columns represent the mean ±SE, \**p* <0.05 *vs.* the colony count at day 0.

**Figure 4. Asses****sments of antibiotic activity and bone regeneration.** (**A**) The results of WBC and CRP in rabbit serum at the time points of 2, 4, 6 and 8 weeks after implantation of VCS and VCS-AB, the points represent the mean ±SE, #*p* < 0.05 and \**p* < 0.05 compared to the model group. (**B**) The coronal section images of tibia analyzed by micro-CT. (**C**) The location of ROI. (**D**) The histograms show the bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecula number (Tb.N), and trabecular separation (Tb.Sp) scores of ROI from five rabbits per group. The columns represent the mean ±SE, \**p* < 0.05 *vs.* the control group or the model group.

**Figure 5. The timeline of all the procedures.**

**DISCUSSION:**

In the previous studies, various kinds of animal models were constructed to study both acute and chronic bone infection; however, the search for the ideal model still persists17,18. In addition, the ideal bone infection model is expected to simulate the pathological characteristics of bone infection in clinical setting, while the modelling periods, remain low cost and easy to carry out. So far, the rabbit bone infection model is the most common model in inflammatory bone disease research, as the rabbits are available, feasible and inexpensive. In our previous study, we compared the death rate and infection rate of rabbits with varying body weights. The results showed that the body weight should be more than 3 kg; otherwise, there would be a high death rate or a high incidence of haematosepsis and a higher mortality rate after surgery.

Unlike earlier studies, the rabbit bone infection models and antibiotic treatment in our study are consistent with the pathological characteristics of human disease and the surgical therapy. In the previous study, animals injected with sodium morrhuate and *S. aureus* did not have pathologic status more than 60 days. Furthermore, the death rate was more than 20%12,19. The overflow *S. aureus* solution from bone defects had been proven to induce a low infection rate. We used bone wax to fill the 2 mm hole on the tibia, in order to block the *S. aureus* solution in the bone marrow and ensured that the holes were full of bone wax by checking the hole with or without blood overflow. As the thickness of a rabbit tibia was 2 mm, we pressed a cylinder of 2 mm diameter and 2 mm height bone wax into the 2 mm diameter holes, which ensured the bone wax filled the hole and could not infiltrate into the bone marrow. Moreover, as the bone wax was flexible and stable, it filled the holes and could not melt or react with bone marrow. In our study, at the 28th day after infection, the bone wax was still complete and filled the holes fully. As the weights of rabbits were more than 3 kg and less than 3.2 kg, the volume of bacteria suspension was 900 µL to 960 µL. Because of the slow speed of injection and blocking function of bone wax, this volume of bacteria suspension could be injected into bone marrow without high pressure. The results showed that this protocol ensures the quantity of *S. aureus* infected in bone marrow. A 2 mm diameter hole was punched in the tibia to ensure that the distance to the upper end of the tibia is 1.5 cm, which locates the hole at the tibial plateau, ensuring sufficient space to debride and implant VCS beads and autogenous bone in the following treatment. During the modelling process, 3 rabbits died because of serious infection. The remained rabbits identified as bone infection rabbits, and the infection rate in the remaining rabbits was 100%. Compared with other bone infection protocols, such as the implantation of sponges soaked with *S. aureus* or the implantation of foreign matter, our protocols closely simulate bone infection in clinical setting, and have little effect on procedures, such as debriding necrotic bone and antibiotic treatment.

Diagnosing bone infection is a challenge to surgeons. The laboratory test results, including serum inflammation marker detection, microbiology analysis and histopathology analysis were used to evaluate bone infection in clinical settings20. Also, diagnostic imaging, such as ultrasound, radiology, computed tomography, magnetic resonance imaging or Raman spectroscopy were applied to detect bone infection21. Unfortunately, diagnosing osteomyelitis through imaging methods is often delayed because bone necrosis is difﬁcult to detect by plain radiography until week 3 of infection. In our study, we used serum inflammation marker detection and histopathology analysis to evaluate bone infection models, as these methods were effective, operable and the indexes were sensitive. The following were the most important steps of the surgical procedure to create a bone infection model in our study. Choose the rabbits with appropriate body weight to perform the surgery and treatment. Maintain a sterile environment during the raising and surgical procedure, and ensure warm conditions following the surgical procedure protocol. Punch a 2 mm diameter hole in the tibia, and ensure the distance to the upper end of the tibia is 1.5 cm. Fill the hole with bone wax, and sew up the periosteum and skin in order to block the bacteria solution. The most important steps of antibiotic treatment are the following. Ensure the pathological bone infection of the rabbits by detecting WBC in whole blood and CRP in the serum. Debride necrotic bone completely, punch two adjacent 4 mm diameter holes and scrape and clean the bone tissue between the 2 holes. Implant 4 pieces of VCS beads and 8 pieces of autogenous bone into bone marrow and bone defect.

After antibiotic treatment, we observed that the rabbits in the VCS-AB group had a higher osteogenic potential than that in the VCS group. This may be because the autogenous bone contains activated osteocytes and bone formation growth factors, which produce bone matrix throughout the graft surface, whereas degradation of VCS induces scarce bone matrix at the defect region. Our results indicate that the autogenous bone has the superior osteogenic capability. Although autogenous bone harvesting is limited in volume and entails morbidity to the donor site, the importance of autogenous bone is not negligible22,23,24. In our study, the autogenous bone was acquired from the tail bone, which avoids systemic injury, and decreases death rate in comparison to gaining autogenous bone from iliac bone. Autograft bone from the tail bone is might be the preferred grafting material in the animal study of bone infection.

In conclusion, an improved rabbit model of bone infection was established in this study. Inflammation indexes and blood biochemical indexes were used to estimate the bone infection model. Also, after antibiotic treatment, multi-level analyses were carried out to detect antibiotic activity and bone regeneration capability. The modeling, treatment protocols and evaluation methods are feasible and reliable. Further studies will be focused on taking advantage of multi-modality visual devices to monitor the pathologic process of bone infection and the bone repair process.

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**DISCLOSURES:**

The authors report no conflicts of interest in this work.

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